University of Alberta

RETROGRADE SIGNALING MECHANISMS OF NERVE GROWTH FACTOR REGULATING THE SURVIVAL AND APOPTOSIS OF SYMPATHETIC NEURONS

by

Sue-Ann Sui-Ah Mok

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in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

Department of Cell Biology

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Dr. Robert B. Campenot, Dept. of Cell Biology

Dr. Paul Melançon, Dept. of Cell Biology

Dr. Richard A. Rachubinski, Dept. of Cell Biology

Dr. Kathryn Todd, Dept. of Psychiatry

Dr. David R. Kaplan, Dept. of Molecular Genetics, University of Toronto

DEDICATION

To all the science underdogs who dare to seek out what is true

ABSTRACT

During development, the survival of neurons is strictly regulated; only 50% of the neurons initially produced during development survive. The survival of several neuron populations during development, including sympathetic neurons, is regulated by neurotrophins such as nerve growth factor (NGF) released from innervation targets. NGF activates its receptor, TrkA, at axon terminals, to generate signals that are transmitted retrogradely to the cell bodies to induce signaling cascades regulating survival. A general view of this process is that NGF generates retrograde survival signals that, when delivered to cell bodies, induce downstream survival signaling that prevents apoptosis. A retrograde survival signal proposed to be necessary for sympathetic neuron survival consists of endosomes containing NGF and phosphorylated TrkA. For this signal, phosphorylated TrkA arriving at the cell bodies is required to initiate survival signaling. Studies have tested the necessity of TrkA phosphorylation in the cell bodies for survival: results from different studies contradict each other. Moreover, the Trk inhibitor, K252a, used in these studies, has reported non-specific effects. Using an alternate Trk inhibitor, Gö6976, data presented in this thesis demonstrates that NGF can promote survival by retrograde signaling that does not require TrkA phosphorylation in the cell bodies. These retrograde signals may be composed of signaling molecules activated downstream of TrkA in axons since pro-survival molecules downstream of TrkA, Akt and CREB, were found activated in the cell bodies/proximal axons.

Data presented in this thesis also reveals the existence of a fundamentally different mechanism for how NGF promotes sympathetic neuron survival: a retrograde apoptotic signal that is suppressed by NGF. NGF withdrawal from axons induced the

"axon apoptotic signal" that was retrogradely transmitted to the cell bodies to activate a key pro-apoptotic molecule, *c*-jun. The axon apoptotic signal, which was blocked by the kinase inhibitors rottlerin and chelerythrine, was necessary for apoptosis in response to NGF deprivation. Evidence GSK3 is involved in the generation or transmission of the axon apoptotic signal was provided by experiments with GSK3 inhibitors and siRNA. The discovery of the axon apoptotic signal refutes the previous view that NGF acting on axon terminals supports survival exclusively by generating retrograde survival signals. The existence of the axon apoptotic signal has broad implications for understanding not only nervous system development but also other conditions where neuronal apoptosis occurs, such as those found in neurotrauma and neurodegenerative diseases.

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LIST OF SYMBOLS, ABBREVIATIONS AND NOMENCLATURE

Akt	Ak transforming
anti-NGF	NGF antibody
APPL1	Adaptor protein, phosphotyrosine interaction, PH domain and leucine zipper containing 1
CB/PAx	Cell Bodies/Proximal Axons
c-jun	Cellular Jun
CREB	cyclic AMP response element binding protein
DAx	Distal Axons
DMSO	dimethyl sulfoxide
DRG	Dorsal root ganglion
Erk	Extracellular signal-regulated kinase
GSK3	Glycogen Synthase Kinase-3
IAx	Intermediate axons
JIP	*c*-jun N-terminal kinase-interacting protein
JNK	*c*-jun N-terminal Kinase
LiCl	lithium chloride
MKK	Mitogen-activated protein kinase kinase
MLK	Mixed lineage kinase
NGF	Nerve Growth Factor
p75NTR	p75 neurotrophin receptor
PI3K	Phosphoinositide 3-kinase
PKC	Protein kinase C
PKCδ	Protein Kinase C delta
POSH	Plenty of SH3 domains
Rac	Ras-related C3 botulinum toxin substrate
SCG	superior cervical ganglion
TrkA	Tropomyosin-related kinase A
αNGF	NGF antibody

CHAPTER 1:

INTRODUCTION

1.1 Neurotrophins and nervous system development

During development, 50% of the neurons generated are subsequently removed by a process of programmed cell death (Oppenheim, 1991). This phenomenon is widespread, affecting many neuronal populations in both the central and peripheral nervous system. In many cases, the neurons have already extended their axons all the way to their targets of innervation, only to be eliminated soon after. How is this process controlled and what dictates which neurons live or die? It was found that a group of proteins called neurotrophic factors promote the survival of neurons during development. Several of these factors, which are structurally related, occur in mammals, and are collectively called neurotrophins: nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and neurotrophin-4/5 (NT-4/5). The first of these neurotrophins to be isolated was NGF (Cohen et al., 1954). Mice lacking NGF displayed dramatic loss of neurons in the superior cervical ganglia (SCG), dorsal root ganglia (DRG), and trigeminal ganglia of the peripheral nervous system (Crowley et al., 1994), indicating that NGF is critical for supporting the survival of specific populations of neurons during development. Deletion of other neurotrophin family members confirmed that they also promote survival of specific populations of neurons during development (reviewed by Huang and Reichardt, 2001). However, these deletion studies also provided evidence that several neurotrophins could contribute to the survival of the same population of neurons, thereby adding a layer of complexity to survival regulation. More complexity is added by the findings that in some neurons, neurotrophins may also induce apoptosis (reviewed by Rabizadeh and Bredesen, 2003). In addition to regulating neuronal survival, neurotrophins are also implicated in other processes during development including proliferation of neuronal precursor cells, differentiation, axon and dendrite growth, axon pruning, and synapse formation (reviewed by Bibel and Barde, 2000; Huang and Reichardt, 2001; Markus et al., 2002). Therefore, neurotrophins are key factors shaping the developing nervous system. Neurotrophins continue to be expressed in adulthood and function in processes such as pain perception, memory formation, and protection from neurodegeneration (reviewed by Fumagalli et al., 2008; Lu et al., 2008; Pezet and McMahon, 2006). Thus, elucidating the molecular mechanisms of neurotrophin signaling has broad consequences for understanding nervous system function.

How do neurotrophins regulate survival during development? For neurons dependent on NGF for survival, it was found that NGF is secreted from the target tissues that the neurons innervate (Korsching and Thoenen, 1983) and that these targets are critical for supporting the survival of these neurons *in vivo* (Hamburger and Levi-Montalcini, 1949). Since target-secreted NGF is only concentrated at the axon terminals innervating the target tissue, it was thought that NGF's survival-promoting signal has to be communicated from the axon terminals to the cell bodies in order to exert its effects. Elucidating the mechanisms for how neurotrophic factors such as NGF can produce signals that retrogradely travel over long distances to the cell bodies to mediate outcomes such as survival, may be highly relevant for understanding other processes involving axon responses to the environment, including deleterious conditions imposed by neurotrauma and neurodegenerative diseases.

1.2 Nerve growth factor (NGF)

Nerve growth factor (NGF) was the first neurotrophic factor and neurotrophin to be isolated and characterized. The actions of NGF were first recognized through studies on an unusual source: mouse sarcoma tissue. It was found that grafting tissue from mouse sarcoma onto chick embryos resulted in an increase in the size of the dorsal root ganglia innervating it (Bueker, 1948). When these experiments were repeated by Levi-Montalcini and Hamburger, they found an increase in size of both the dorsal root ganglia and the sympathetic ganglia innervating the tumour, but they also made the fortuitous observation that even ganglia that did not directly innervate the tumour were also increased in size (Levi-Montalcini and Hamburger, 1951). This suggested to the investigators that the effects were not a result of direct contact with the tumour, but rather a result of a soluble diffusible factor that was secreted from the tissue and could come in contact with unconnected neurons. When the tumour was grafted to chick embryos at the chorioallantoic membrane, which is connected to the vasculature network but has no other connection with the chick itself, a similar increase in the size of ganglia was observed (Levi-Montalcini, 1952 ; Levi-Montalcini and Hamburger, 1953). *In vitro* experiments showed that the tumour sarcoma tissue potently stimulated neurite outgrowth of co-cultured sympathetic and dorsal root ganglion explants (Levi-Montalcini et al., 1954). These *in vitro* studies led to the isolation of the factor that would later be named nerve growth factor (NGF), when it was accidentally discovered that snake venom also contained potent growth stimulating properties (Cohen, 1960; Cohen and Levi-Montalcini, 1956). Snake venom is produced by a salivary gland and NGF was eventually isolated from male mouse submaxillary salivary gland, in 1960 (Cohen, 1960).

NGF is synthesized as a 30-kDa proneurotrophin precursor, proNGF, that is subsequently cleaved by furin, proconvertases, or matrix metalloproteinases to the mature 13 kDa NGF (Bresnahan et al., 1990; Edwards et al., 1988; Heymach and Shooter, 1995;

Lee et al., 2001). Following processing, the mature NGF exists as a non-covalent dimer (Angeletti et al., 1973a; 1973b). The other members of the neurotrophin family expressed in mammals have been designated brain derived neurotrophic factor (BDNF) (Barde et al., 1982; Leibrock et al., 1989), neurotrophin-3 (NT-3) (Hohn et al., 1990; Jones and Reichardt, 1990; Maisonpierre et al., 1990), and neurotrophin-4/5 (NT-4/5) (Berkemeier et al., 1991; Hallbook et al., 1991). All the neurotrophins are structurally related, containing a characteristic protein motif called a cysteine knot that is formed by three disulfide bridges (reviewed by Wiesmann and de Vos, 2001). As stated previously, neurotrophins regulate diverse processes during development and in adulthood. Recently, it has been found that the precursor forms of neurotrophins, proneurotrophins, may also have biological functions in neurons. However, in contrast to the survival function generally ascribed to neurotrophins, proneurotrophins promote apoptosis (Lu et al., 2005).

A role for NGF in promoting survival during development has been demonstrated in NGF knockout mice which display an almost complete loss (>95%) of sympathetic neurons as well as loss of the majority of sensory neurons in the dorsal root and trigeminal ganglia. NGF is secreted from the target tissues that these neurons innervate (Korsching 1983, Davies 1987) suggesting that NGF promotes survival after the neurons have extended their axons to their targets.

1.3 Neurotrophin receptors

Neurotrophin actions are initiated by their binding to receptors. The first neurotrophin receptor to be cloned, p75NTR (Chao et al., 1986; Radeke et al., 1987), binds all neurotrophins with equal affinity (Frade and Barde, 1998; Rodriguez-Tebar et al., 1990; 1992; Timm et al., 1994). p75NTR also binds proneurotrophins (Lee et al., 2001). In contrast, a second group of neurotrophin receptors, which are members of the tropomyosin-related kinase (Trk) family, are more selective for neurotrophin ligand binding. Their name derives from the original identification of the first Trk, TrkA, as a proto-oncogene of a fusion protein consisting of tropomyosin and an unidentified tyrosine kinase (Kaplan et al., 1991a). TrkA preferentially binds NGF, although it can also bind NT-3 at higher concentrations. TrkB binds to both BDNF and NT-4/5 and again NT-3 at higher concentrations, and TrkC binds NT-3 (for review see Barbacid, 1994). The Trk receptors bind to proneurotrophins with a relatively poor affinity compared to p75NTR in neurons (Lee et al., 2001). The greater affinity of proneurotrophins for p75NTR is likely due to the formation of a complex between p75NTR and a co-receptor, sortilin (Nykjaer et al., 2004).

1.3.1 TrkA

TrkA is the preferential Trk receptor for NGF suggesting it may mediate NGF actions. In support of TrkA's role in regulating NGF-dependent survival, neurons that are regulated by NGF during development express TrkA during the period of NGF-dependent survival (Fagan et al., 1996), and targeted disruption of the TrkA gene leads to the loss of the same neuronal populations that are absent in NGF knockout mice (Smeyne et al., 1994).

TrkA is a receptor tyrosine kinase with a molecular weight of 140 kDa (Kaplan et al., 1991a). Its extracellular domain consists of a cysteine-rich cluster followed by three leucine-rich repeats, another cysteine-rich cluster and two immunoglobulin-like domains (Schneider and Schweiger, 1991). The immunoglobulin-like domains have been shown to be sufficient for NGF to bind to TrkA (Perez et al., 1995). However, other studies have shown that the second leucine-rich repeat may also contribute to neurotrophin binding (Windisch et al., 1995a; 1995b). The extracellular domain is connected to the intracellular domain by a single transmembrane segment. The intracellular domain contains a tyrosine kinase domain and several tyrosine residues that are phosphorylated upon receptor activation (Inagaki et al., 1995; Stephens et al., 1994).

1.3.2 p75NTR

Although studies with knockout mice have demonstrated that TrkA is critically important for promoting the survival of neurons that are dependent on target-derived NGF during development, it has also been recognized that p75NTR, which is also expressed during development (Wyatt and Davies, 1993; 1995), regulates the survival of these neurons as well. In contrast to TrkA, p75NTR is a receptor associated with promoting apoptosis. Mice with target disruption of p75NTR showed a significant delay in apoptosis of sympathetic neurons of the SCG during the normal period of developmental death(Bamji et al., 1998). Sensory neurons of the trigeminal ganglia of p75NTR$^{-/-}$ mice were also protected from developmental apoptosis (Agerman et al., 2000). In apparent conflict with p75NTR's predicted pro-apoptotic function, a decrease in the size of the dorsal root ganglia (DRG) was found in p75NTR$^{-/-}$ mice (Lee et al., 1992). However, it was later found that the increased death of DRG sensory neurons was due to impairment of Schwann cell migration which is a p75NTR-dependent process (Cosgaya et al., 2002; Yamauchi et al., 2004).

p75NTR is classified as a member of the tumor necrosis factor (TNF) superfamily of receptors based on the presence of cysteine-rich repeats in its extracellular domain, but is unique because its neurotrophin and proneurotrophin ligands are structurally unrelated to the ligands normally bound by members of the TNF receptor family (reviewed by Baker and Reddy, 1998). p75NTR has four cysteine-rich repeats that are all required for ligand binding (He and Garcia, 2004). The extracellular domain is connected to its intracellular domain by a single membrane spanning region. The intracellular domain has no known enzymatic activity but does contain several motifs that function as binding sites for adaptor proteins. Identified motifs include two putative TRAF binding sites, a PDZ domain binding site, and a type II death domain (reviewed by Roux and Barker, 2002). p75NTR also contains cleavage sites for γ-secretase in its intracellular domain and α-secretase in its extracellular domain.

There is a growing body of evidence that p7NTR promotes apoptosis by mechanisms involving neurotrophin or proneurotrophin binding to p75NTR that induces the binding of adaptor proteins and activation of downstream apoptotic signaling pathways. In the case of proneurotrophins, p75NTR binding may be facilitated by co-receptors such as sortilin (Nykjaer et al., 2004). In the case of neurotrophins, it has been demonstrated that in NGF-dependent sympathetic neurons, the addition of neurotrophins that do not preferentially bind to TrkA (BDNF, proBDNF, proNGF, NT-4) induces apoptosis (Bamji et al., 1998; Deppmann et al., 2008; Kenchappa et al., 2006; Nykjaer et al., 2004). In support of a role of BDNF in mediating p75NTR-dependent apoptosis, deletion of the BDNF gene in mice resulted in an increase in the number of sympathetic neurons of the SCG during development (Bamji et al., 1998).

Studies in sympathetic neurons have provided evidence that both survival signaling mediated by TrkA and apoptotic signaling mediated by p75NTR regulate the survival of neurotrophin-dependent neurons during development. The loss of sympathetic neurons observed in TrkA$^{-/-}$ mice can be partially rescued by disrupting p75NTR (Majdan et al., 2001). This result suggests that p7NTR does not promote apoptosis by inhibiting TrkA signaling but instead function by inducing pro-apoptotic signaling. Other evidence supports a concept that TrkA signaling mediated by NGF promotes survival by suppressing apoptosis induced by p7NTR signaling. When sympathetic neurons were cultured in 10 ng/ml NGF, addition of BDNF to activate p75NTR signaling had no effect on neuronal survival (Bamji et al., 1998). However, when neurons were cultured in a lower concentration of NGF (2.5 ng/ml), it was found that activation of p75NTR with BDNF resulted in a significant increase in apoptosis. This evidence suggests that sympathetic neuron survival is dependent on TrkA signaling which must be made in sufficient amounts to suppress p75NTR apoptotic signaling.

Since p75NTR regulates sympathetic neuron survival by inducing pro-apoptotic signaling, potential signaling mechanisms activated downstream of p75NTR will be discussed the apoptotic signaling section (Section 1.5.4) later in the Introduction.

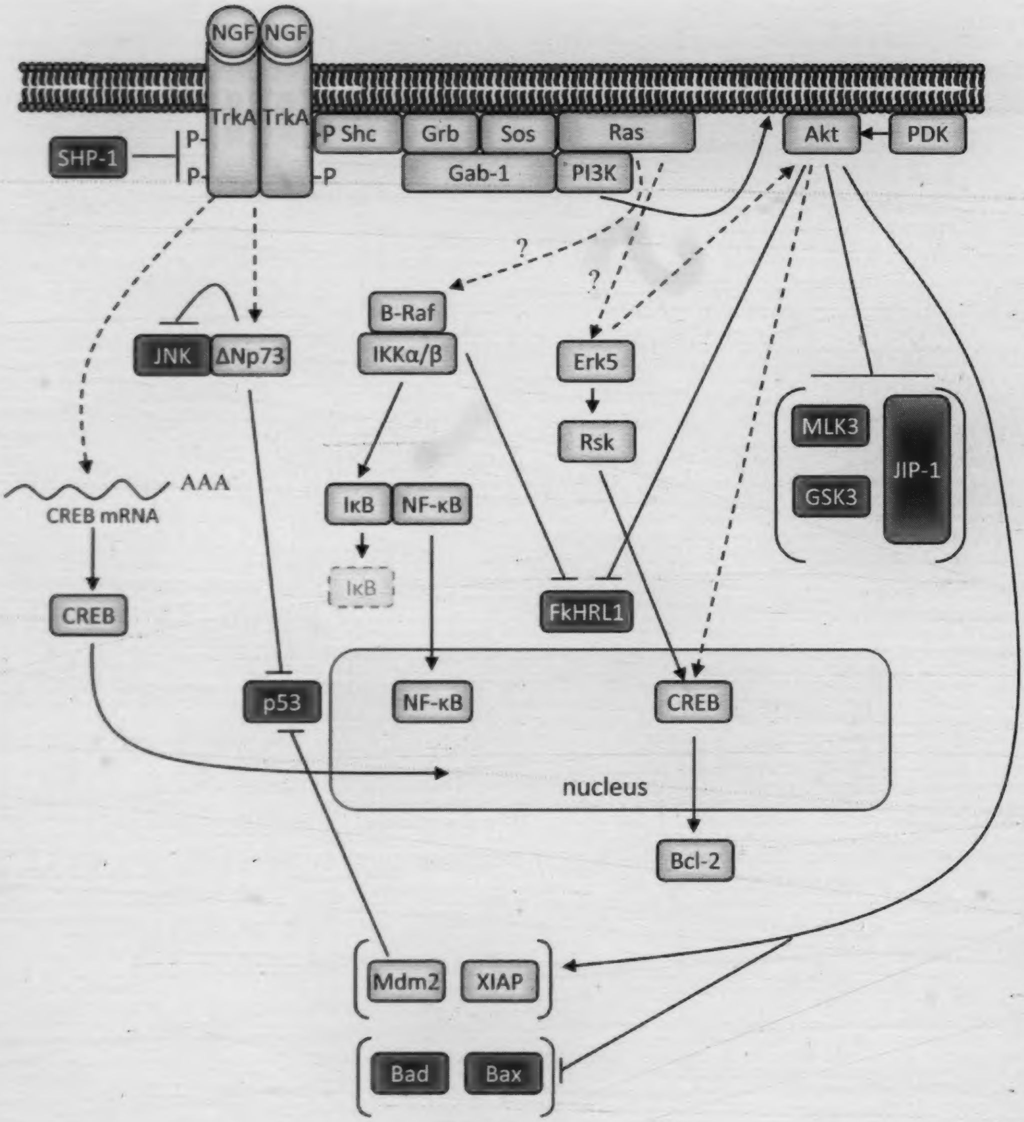
1.4 Survival signaling mechanisms activated by NGF

Most of the molecular characterization of the pathways initiated by NGF have been elucidated using an NGF responsive cell line, PC12, which is derived from a pheochromocytoma (neuroendocrine tumour) of the rat adrenal medulla. In response to NGF, these cells differentiate, extend neurites and become dependent on NGF for their survival in the absence of serum (Greene, 1978; Greene and Tischler, 1976; Tischler and Greene, 1975). These cells provide an excellent source of biological material for carrying out biochemical studies on NGF-activated pathways, but due to the fact that these cells are not neurons, the pathways delineated in this cell culture model must be evaluated in NGF-dependent neurons such as sympathetic or sensory neurons to confirm their relevance. NGF signaling has been implicated in a multitude of processes in neurons including survival, axon growth, and synapse development and function (for review see Bibel and Barde, 2000; Sofroniew et al., 2001). For the purposes of this thesis, the focus will be on the pathways implicated in promoting survival in NGF-dependent neurons. The survival pathways implicated in promoting sympathetic neuron survival have been depicted in a schematic diagram (Figure 1-1). It is important to note that not all signaling events implicated in promoting survival have been characterized with respect to the location of their activity within the neuron (axon terminals, axon, cell bodies). Therefore, where various signaling pathways are activated or how they interact may be dictated by the localization of specific signaling molecules within the neuron.

1.4.1 Activation of TrkA by NGF

NGF can effectively mediate survival through activation of the TrkA receptor. Dimers of NGF bind to the TrkA receptor which leads to the receptor's dimerization and autophosphorylation at multiple residues both within and outside its tyrosine kinase domain (Inagaki et al., 1995; Stephens et al., 1994). Phosphorylation within the kinase domain stimulates its kinase activity (Cunningham and Greene, 1998). There are two major tyrosine phosphorylation sites outside the kinase domain, Y490 and Y785, and their phosphorylation creates docking sites for proteins containing phosphotyrosine-binding (PTB) domains and Src-homology-2 (SH-2) domains, respectively (Obermeier et al., 1993a; 1993b). Their association with TrkA and subsequent phosphorylation by TrkA leads to additional recruitment of proteins and the activation of signaling cascades

Figure 1-1. Survival signaling pathways activated by NGF. NGF-induced dimerization and subsequent autophosphorylation of the TrkA receptor at the plasma membrane result in the activation of multiple downstream signaling molecules/signaling cascades which promote neuron survival. Signaling molecules implicated in promoting NGF-dependent survival are colored green. Signaling molecules implicated in promoting apoptosis in the absence of NGF are colored blue. Dashed arrows connecting two signaling molecules indicate the presence of intermediate signaling events which have not been identified and question marks (?) indicate that the potential upstream regulator has been identified, but not confirmed, to promote the downstream survival pathway in neurons.


NGF
NGF
TrkA
TrkA
P
P
P
P
SHP-1
Shc
Grb
Sos
Ras
Gab-1
PI3K
Akt
PDK
?
?
B-Raf
IKKα/β
JNK
ΔNp73
Erk5
Rsk
MLK3
GSK3
JIP-1
AAA
CREB mRNA
CREB
IκB
NF-κB
IκB
FkHRL1
p53
NF-κB
CREB
nucleus
Bcl-2
Mdm2
XIAP
Bad
Bax

implicated in promoting neuronal survival. Analysis of the related receptor, TrkB, provides evidence that Y490 is the major phosphorylation site required for mediating neurotrophin-dependent survival in sympathetic neurons (Atwal et al., 2000). TrkA activity is subject to negative regulation by Src homology phosphatase-1 (SHP-1). TrkA activates SHP-1 (Vambutas et al., 1995), which in turn promotes its association with the activated receptor and dephosphorylation of tyrosine residues in the receptor's kinase domain (Marsh et al., 2003).

1.4.2 The PI3K/Akt pathway

The phosphoinositide-3 kinase/Ak transforming (PI3K/Akt) pathway is the most well studied NGF survival pathway to date. Two main routes have been described for the activation of PI3K following TrkA activation. In the first route, the scaffold protein Shc is recruited to the activated receptor at the phosphorylated Y490/PTB docking site (NPXpY) and then is phosphorylated by TrkA (Obermeier et al., 1993; Stephens et al., 1994). This leads to further recruitment of another adaptor protein, Grb2, and the guanine nucleotide exchange factor, Sos, which in turn activates the membrane-associated GTPase, Ras (reviewed by Segal and Greenberg, 1996). Activated Ras can then stimulate PI3K activity (Rodriguez-Viciana et al., 1994). In the second route, Shc and Grb2 bind Gab-1, which in turn binds PI3K (Holgado-Madruga et al., 1997). PI3K recruited to the membrane gives it access to phosphoinositols, which it phosphorylates to generate phosphatidylinositol (3,4,5)-trisphosphate (PIP3). PIP3 then recruits Akt to the membrane where it can then be activated by phosphatidylinositide-dependent kinase (PDK-1/2) (reviewed by Vanhaesebroeck and Alessi, 2000). The activated Akt can then go on to phosphorylate downstream targets that promote survival.

The requirements of several components of these pathways have been tested in neuron models. Mice carrying null mutations for two Shc genes, ShcB and ShcC, display a significant loss of sympathetic neurons in the SCG during development (Sakai et al., 2000). Consistent with a pro-survival role for Ras, introduction of Fab fragments of Ras antibodies into sympathetic neurons partially blocked their survival (Nobes et al., 1996), and expression of a constitutively activated form of Ras in sympathetic neurons partially protected them from apoptosis following NGF withdrawal (Mazzoni et al., 1999). The requirement of PI3K for neuronal survival has been examined in numerous studies and the results are divided. Some studies showed that inhibiting PI3K either pharmacologically with LY294002 or by expression of dominant-negative PI3K produced at least a partial block of NGF-dependent survival in sympathetic neurons (Atwal et al., 2000; Crowder and Freeman, 1998; Klesse and Parada, 1998; Tsui-Pierchala et al., 2000; Vaillant et al., 1999), while others could not demonstrate a significant effect (Philpott et al., 1997; Virdee et al., 1999). This discrepancy may be due to differences in the assays used to measure survival. It is possible that PI3K mediates some events associated with survival but not others. The direct requirement for Akt activity for survival has also been tested and in this case the studies are in agreement; expression of dominant-negative Akt in sympathetic neurons does block the survival of neurons supplied with NGF (Crowder and Freeman, 1998; Vaillant et al., 1999; Virdee et al., 1999).

How would activation of the PI3K/Akt pathway promote survival in NGF-dependent neurons? It is likely that Akt mediates its effects by phosphorylating a variety of pro-apoptotic substrates to inhibit their activity. Candidate substrates implicated in sympathetic neuron apoptosis induced by NGF withdrawal include Bad (Datta et al., 1997), Bax (Gardai et al., 2004), FOXO3a (Brunet et al., 1999), MLK3 (Barthwal et al., 2003), ASK1 (Kim et al., 2001), and Mdm2 (Mayo and Donner, 2001). Another possible substrate for Akt in sympathetic neurons is GSK3β (glycogen synthase kinase-3 β; Cross et al., 1995). GSK3β has been implicated as a pro-apoptotic molecule in several neuron models (Chen et al., 2004; Chin et al., 2005; Koh et al., 2005; Perez et al., 2003). However, there is no evidence thus far to support an apoptotic role for GSK3β in sympathetic neurons during NGF withdrawal. In the one study examining GSK3β's roles in sympathetic neuron survival, it was found that exogenous expression of protein inhibitors of GSK3β function could not rescue sympathetic neurons from apoptosis in response to NGF withdrawal, suggesting that GSK3β activity is not an obligatory factor for apoptosis (Crowder and Freeman, 2000). Akt can also inhibit pro-apoptotic molecules via direct binding interactions such as has been found for JIP-1 (Kim et al., 2002).

Akt may also promote survival by activating or stabilizing pro-survival signaling molecules such as XIAP (Dan et al., 2004) or CREB. It has been demonstrated that overexpression of Akt induces CREB-dependent transcription in PC12 cells (Pugazhenthi et al., 2000), but it has not been confirmed if Akt is required for CREB activity in NGF-dependent neurons. It is known that NGF does induce CREB activation in both PC12 cells and sympathetic neurons (Riccio et al., 1997; 1999). In PC12 cells, NGF stimulated the CREB-dependent transcription of the anti-apoptotic molecule Bcl-2 (Riccio et al., 1999). In sympathetic neurons, preventing CREB from binding to its promoter sites blocked NGF-mediated survival, and transfection of these neurons with a constitutively active CREB prevented apoptosis in the absence of NGF (Riccio et al., 1999). Moreover, CREB null mice displayed a loss of sensory neurons in the dorsal root and trigeminal ganglia occurring during the period of neurotrophin dependence (Lonze et al., 2002). These mice also displayed a loss of sympathetic neurons from their SCG, but the death occurred prior to the normal period of NGF dependence and therefore is not evidence that CREB mediates NGF-dependent survival of sympathetic neurons *in vivo*. A recent study by Parlato et al. (2007) does not appear to support a role for CREB in mediating sympathetic neuron survival. In this study, mice were generated with selective deletion of CREB in the noradrenergic and adrenergic neurons, such as sympathetic neurons. The result was an increase in the size of the SCG compared to control mice, suggesting that CREB actually functions as a pro-apoptotic factor in sympathetic neurons (Parlato et al., 2007). It is possible that in mice completely lacking CREB (Lonze et al., 2002), other cells that may be required to correctly target sympathetic neurons or support their early survival are negatively affected by the mutation, leading to a secondary loss of the sympathetic neurons. However, this does not explain the results obtained in experiments with cultured sympathetic neurons, which provide evidence for a pro-survival function for CREB (Riccio et al., 1999). Therefore, it seems that defining CREB's role in neuronal survival will require further investigation.

1.4.3 The NF-KB pathway

Although much of the research has been focused on characterizing how survival is promoted by the PI3K/Akt pathway, there is mounting evidence that other pathways activated by NGF contribute to this process. Maggiwar et al. (1999) implicated the transcription factor complex, NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells), in promoting NGF-dependent survival of sympathetic neurons in culture. In studies looking at both Akt and NF-κB, it was found that these two proteins prevented different aspects of the apoptotic program in sympathetic neurons, suggesting that they promote survival through independent pathways (Sarmiere and Freeman, 2001). NF-κB has been shown to promote the gene expression of anti-apoptotic proteins such as members of the Bcl-2 (B cell lymphoma-2) and IAP (inhibitor of apoptosis protein) families (reviewed by Kucharczak et al., 2003) in certain cell types but not in NGF-dependent neurons as of yet. NF- κB activity is regulated by IκB (inhibitor of κB) which binds NF-κB and sequesters it in the cytoplasm. Nuclear translocation of NF-κB requires proteasomal degradation of IκB that is triggered when IκB is phosphorylated by IκB kinase (IKK). Recently, it was shown that IKK, composed of the subunits IKKα and IKKβ, was required for NGF-dependent survival of cultured sympathetic neurons (Encinas et al., 2008). IKK functions upstream of NF-κB, but may also act by phosphorylating other apoptotic targets such as FkHRL1/FOXO and preventing their function (Hu et al., 2004). Upstream of IKK, it was found that IKK activation in response to NGF required B-Raf and that B-Raf was also required for NGF-dependent survival (Encinas et al., 2008). B-Raf is a known downstream effector of Ras, and it is possible that Ras activation downstream of TrkA may play a role in promoting IKK/NF-κB signaling. However, this needs to be verified, since a previous study showed that expression of a Ras mutant that activated B-Raf but not PI3K in sympathetic neurons produced only a small amount of survival (Mazzoni et al., 1999).

1.4.4 ΔNp73

Knockout mice have provided evidence for a role of the p53 family member, ΔNp73, in promoting the survival of sympathetic neurons during development (Pozniak et al., 2000). Overexpression of ΔNp73 in sympathetic neurons in the absence of NGF had several anti-apoptotic effects, including 1) preventing expression of apoptotic proteins p21Waf1, Apaf-1, and Bim1, 2) blocking cytochrome *c* release and 3) inhibiting *c*-jun phosphorylation possibly by directly interacting with JNK (Lee et al., 2004). Genetic evidence suggests that ΔNp73 may mediate some of its effects by inhibiting the pro-apoptotic transcription factor, p53, thereby preventing p53-dependent expression of p21Waf1 and Apaf-1 (Lee et al., 2004). ΔNp73 is regulated by degradation; ΔNp73 protein levels decrease in response to NGF withdrawal in sympathetic neurons (Pozniak et al., 2000).

1.4.5 Erk5

A recent study shows that deletion of Erk5 in cultured sympathetic neurons results in a small but significant decrease in survival (Finegan et al., 2009). A previous study in sensory neurons also supported a role for Erk5 in mediating neurotrophin survival signaling possibly through downstream activation of CREB (Watson et al., 2001). Finegan et al. suggested that Erk5 may promote survival by inducing Akt-dependent inhibition FkHRL1/FOXO3a and Rsk-dependent activation of CREB. It should be noted that NGF-induced activation of Erks by a Ras-Raf-Mek-Erk signaling cascade in sympathetic and sensory neurons had been previously recognized, and several studies carried out with general MEK inhibitors or expression of mutants that blocked the Ras-Raf-Mek-Erk pathway did not show a significant reduction in the ability of NGF to mediate survival (Creedon et al., 1996; Klesse and Parada, 1998; Mazzoni et al., 1999; Virdee and Tolkovsky, 1995). However, given the data provided by Finegan et al. (2009)

and Watson et al. (2001), the possibility that Erk5 contributes to NGF-dependent survival cannot be ruled out at this time.

1.4.6 NGF-stimulated translation of CREB

In sensory neurons it was found that NGF stimulated the local translation of CREB mRNA in axons. The newly synthesized CREB in axons was then retrogradely transmitted to the cell bodies to support survival (Cox et al., 2008). How signaling downstream of NGF stimulates local translation of CREB has not been determined, but it raises the interesting possibility that other mRNAs may also be translated in axons, in response to NGF, to promote survival.

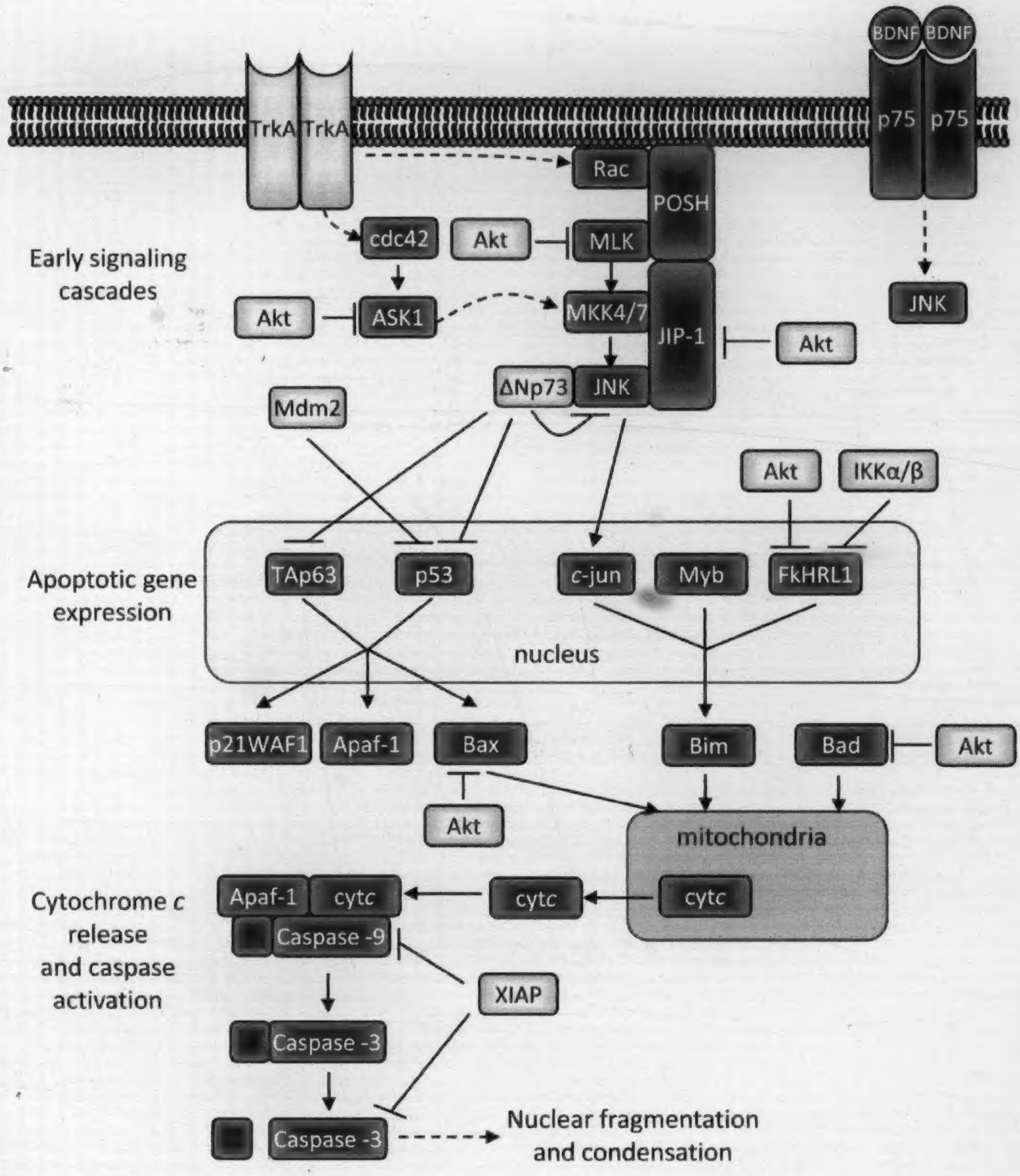
1.5 Apoptotic signaling in response to NGF deprivation

Cultured sympathetic neurons isolated from the SCG of newborn rats and cultures of differentiated PC12 cells have been the main models used to study the apoptotic program initiated by NGF withdrawal. Roughly, the apoptotic program can be divided into a series of stages: activation of early signaling cascades, transcription of pro-apoptotic genes, cytochrome *c* release from mitochondria leading to caspase activation, and nuclear fragmentation and condensation. A schematic diagram of apoptotic events initiated by NGF withdrawal is provided in Figure 1-2.

1.5.1 Activation of early signaling cascades

One of the earliest indications of apoptosis following NGF withdrawal is the activation of the *c*-jun N-terminal kinase (JNK) signaling cascade. In sympathetic neurons, this cascade involves the sequential activation of MLKs/ASK1, MKK4/7, and JNKs. Multiple members of the MLK (mixed lineage kinase) family and ASK1 (apoptosis signal-regulating kinase-1) are expressed in sympathetic neurons, and

Figure 1-2. Apoptosis program initiated by NGF withdrawal. Loss of NGF signaling initiates an apoptotic program which can be divided into several stages: activation of early signaling cascades, pro-apoptotic gene expression, and cytochrome *c* release from mitochondria leading to caspase activation, and subsequent DNA fragmentation and nuclear condensation. Signaling molecules implicated in promoting apoptosis in the absence of NGF are colored blue. Signaling molecules which are activated by NGF signaling and inhibit specific apoptotic molecules are also included for reference and are colored green. Dashed arrows connecting two signaling molecules indicate a requirement for intermediate signaling events which have not been identified.


BDNF
BDNF
TrkA
TrkA
p75
p75
Rac
POSH
cdc42
Akt
MLK
Early signaling cascades
Akt
ASK1
MKK4/7
JIP-1
Akt
JNK
ΔNp73
JNK
Mdm2
Akt
IKKα/β
Apoptotic gene expression
TAp63
p53
c-jun
Myb
FkHRL1
nucleus
p21WAF1
Apaf-1
Bax
Bim
Bad
Akt
Akt
mitochondria
Cytochrome c release and caspase activation
Apaf-1
cytc
cytc
cytc
Caspase -9
XIAP
Caspase -3
Caspase -3
Nuclear fragmentation and condensation

transfection of neurons with dominant negative mutants of either the MLK family members, DLK and MLK3, or ASK1 prevented apoptosis in response to NGF withdrawal (Kanamoto et al., 2000; Xu et al., 2001). Of the two MKKs (mitogen-activated protein kinase kinases), it has been shown that expression of dominant-negative MKK4 (Eilers et al., 1998) did not protect sympathetic neurons from apoptosis, which could indicate that either MKK7 is the main kinase for JNKs or that the MKKs are functionally redundant for activating JNKs in this system. There are three JNK genes that through differential splicing generate up to 12 isoforms in total (reviewed by Kyriakis and Avruch, 2001). Of the three genes, JNK3 has been implicated in sympathetic neuron apoptosis by studies in JNK3-deficient mice (Bruckner et al., 2001).

Another early signaling event commencing shortly after NGF withdrawal is the activation of the GTPases, Rac1 (Ras-related C3 botulinum toxin substrate 1) and Cdc42 (cell division cycle 42). Transfection of sympathetic neurons with dominant negative mutants of either Rac1 or Cdc42 inhibited apoptosis. Genetic studies in PC12 cells placed Rac and Cdc42 upstream of MLKs (Xu et al., 2001) and Cdc42 upstream of ASK1(Kanamoto et al., 2000). How loss of NGF signaling leads to activation of Rac and Cdc42, and the mechanism by which these proteins induce the activation of MLKs and ASK1 in sympathetic neurons have not been determined.

Another feature of the early apoptotic signaling cascades is their possible assembly into complexes by scaffolding proteins. Scaffolding proteins function by binding multiple proteins to enable their interaction. Two scaffold proteins, POSH (Plenty of SH3 domains) and JIP-1 (JNK-interacting protein-1), have been linked to this signaling pathway. POSH was originally identified as in interactor with Rac-GTP (Tapon et al., 1998). *In vitro*, POSH was found to also bind MLK3, and JIP-1 (Kukekov et al., 2006). JIP-1 in turn bound MKK4/MKK7 and JNK1. The interaction of POSH with MLK3, MKK7, and JIP-1 in a complex was also confirmed in PC12 cells. The ability of scaffold proteins such as POSH and JIP-1 to bring several members of the same pathway into close proximity might serve to regulate the activation or duration of signals or deliver the signaling complexes to specific sub-cellular locations (reviewed by Morrison and Davis, 2003). A possible role for POSH function in promoting apoptosis of sympathetic neurons has been suggested by the finding that introduction of antisense oligos against POSH partially prevented apoptosis induce by NGF deprivation (Xu et al., 2003). One of the major downstream targets of activated JNKs is the transcription factor *c*-jun which is implicated in apoptotic gene expression.

1.5.2 Apoptotic gene expression

The importance of gene expression in promoting apoptosis of sympathetic neurons was provided by a study showing that inhibiting either transcription or translation prevented apoptosis in response to NGF deprivation (Martin et al., 1988). The first transcription factor shown to be activated during NGF withdrawal was *c*-jun (Virdee et al., 1997). JNK phosphorylates *c*-jun at its Ser63 and Ser73 residues, which stimulates *c*-jun's transcription promoting activity (Pulverer et al., 1991; Smeal et al., 1991). Inhibition of the JNK pathway in sympathetic neurons prevented *c*-jun activation, suggesting that JNK is the primary activator of *c*-jun (Eilers et al., 2001; Harding et al., 2001; Harris et al., 2002). The function of *c*-jun is required for apoptosis of sympathetic neurons in response to NGF withdrawal as evidenced by the lack of apoptosis observed when *c*-jun was deleted in these cells (Palmada et al., 2002). Two other transcription factors, Myb and FkHRL1/FOXO3a, are also activated in response to NGF deprivation (Gilley et al., 2003; Liu et al., 2004), although the upstream pathways for these proteins have not been defined in sympathetic neurons. What are the targets of these transcription factors? One common target shared by these transcription factors appears to be Bim (Bcl-2 interacting mediator of cell death). The Bim promoter has binding sites for *c*-jun, Myb, and FkHRL1/FOXO3a, and there is evidence that all three transcription factors must be present to induce Bim expression during NGF deprivation (Biswas et al., 2007).

Yet another set of transcription factors activated by NGF deprivation are p53, and a related family member, TAp63. Deletion of either TAp63 or p53 prevented the apoptosis of sympathetic neurons *in vivo* (Aloyz et al., 1998; Jacobs et al., 2005) and for TAp63, in cultured sympathetic neurons deprived of NGF as well (Jacobs et al., 2005). Although it has not been confirmed how these proteins become activated in response to NGF deprivation, there is evidence that loss of binding and ubiquitination of p53 by Mdm2 stabilizes p53 levels, an event that can be mediated by phosphorylation of p53 by JNK (Fuchs et al., 1998a; 1998b) . Once activated, TAp63 and p53 induce expression of the pro-apoptotic genes Apaf-1, Bax, and p21WAF1 (Jacobs et al., 2005).

1.5.3 Cytochrome *c* release from mitochondria leading to caspase activation

The release of cytochrome *c* is likely regulated by the activities of molecules such as Bax and Bim, which are known mediators of this process (for review see Willis and Adams, 2005). In support of a role for Bax, deletion of Bax in mice protected sympathetic neurons from developmental apoptosis (Deckwerth et al., 1996). It is also probable that loss of NGF-mediated Bcl-2 expression (Riccio et al., 1999) contributes to cytochrome *c* release.

Release of cytochrome *c* from mitochondria allows the formation of an "apoptosome complex" containing cytochrome *c*, Apaf-1, and caspase-9 (Riedl and Salvesen, 2007). Formation of the complex allows for the activation of caspase-9 that can then carry out the activating cleavage of caspase-3. Evidence that this apoptotic signaling cascade operates in sympathetic neurons is that deletion of Apaf-1, caspase-9, or caspase-3 prevented apoptosis of cultured sympathetic neurons in response to NGF deprivation even though cytochrome *c* release still occurred (Wright et al., 2007).

Once caspases are activated, NGF can no longer rescue sympathetic neurons from apoptosis and the subsequent fragmentation of DNA and condensation of the nucleus marks the endpoint of the apoptotic program (Deshmukh et al., 1996).

1.5.4 A role for p75NTR in promoting apoptosis induced by NGF deprivation

As previously mentioned, activation of p75NTR in sympathetic neurons by neurotrophins other than NGF induces signaling that promotes apoptosis (Bamji et al., 1998; Deppmann et al., 2008; Kenchappa et al., 2006; Nykjaer et al., 2004). However, in the experiments performed in this thesis, when cultured neurons are deprived of NGF, no other neurotrophins have been exogenously added to the culture medium to stimulate p75NTR activation which leads one to question if p75NTR signaling is contributing to the apoptosis under these conditions. Sympathetic neurons synthesize the p75NTR ligands BDNF, NT-3, and NT-4/5 (Causing et al., 1997; Roosen et al., 2001; Schecterson and Bothwell, 1992). Experiments in which BDNF antibody added to cultured sympathetic neurons antagonizes p75NTR mediated processes such as inhibition of axon growth (Kohn et al., 1999) and axon pruning (Singh et al., 2008) is interpreted as evidence that endogenous BDNF secreted from cultured neurons acts in an autocrine manner to activate p75NTR signaling. Based on these studies, it is plausible that secreted BDNF may be promoting p75NTR apoptotic signaling in NGF-deprived neurons in culture.

Since p75NTR lacks enzymatic activity, signaling by p75NTR is initiated by the recruitment of adaptor proteins to p75NTR's intracellular domain stimulated by neurotrophin binding. The interaction of p75NTR with several of these adaptor proteins (NRAGE, NRIF, TRAF6) has been shown to lead to the activation of JNK (Linggi et al., 2005; Salehi et al., 2002; Yeiser et al., 2004). Mice deficient for these adaptors showed delayed developmental death of sympathetic neurons in the SCG, similar to p75NTR$^{-/-}$ mice (Bertrand et al., 2008; Casademunt et al., 1999; Yeiser et al., 2004). Experiments done in culture show that NRIF$^{-/-}$ sympathetic neurons are not protected from apoptosis in response to NGF deprivation suggesting it does not mediate a potential p75NTR apoptotic signal under the conditions explored in this thesis (Linggi et al., 2005). The signaling events linking adaptor protein binding to JNK activation are generally unclear, but for NRAGE in PC12 cells, signaling appears to be independent of MLK (Salehi et al., 2002) which has been implicated in NGF withdrawal induced activation of JNK (see Section 1.5.1). Rac has been demonstrated to mediate JNK in response to p75NTR activation in oligodendrocytes (Harrington et al., 2002), and since Rac has not been shown to bind directly to p75NTR, it is hypothesized that Rac is activated downstream of adaptor binding. The components lying between Rac and JNK in the p75 signaling pathway have not been elucidated. In addition to activation of JNK, p75NTR may also induce signaling through its proteolytic cleavage which generates an intracellular domain fragment that is capable of delivering bound adaptors to their nuclear targets (Kenchappa et al., 2006).

One issue that needs to be addressed when considering if p75NTR is involved in apoptotic signaling following NGF deprivation is the study by Palmada et al. (2002) that provides evidence that c-jun is required for apoptosis in response to NGF withdrawal but not activation of p75NTR by BDNF addition. These results suggest that p75NTR is not involved in NGF deprivation–induced apoptosis because it seems to activate a different, c-jun-independent, pathway. However, in this study and many of the other studies exploring BDNF-stimulated p75NTR apoptotic signaling, the neurons are maintained under depolarizing conditions. These depolarizing conditions are used to promote the survival of the neurons in the absence of NGF, which has been removed to prevent any possible complications of NGF signaling through p75NTR. The signaling pathways activated by depolarizing conditions to keep the neurons alive (Vaillant et al., 1999) may inhibit signaling events of the apoptotic program which occur normally under conditions of NGF deprivation. In addition, the high concentrations of BDNF (100-200 ng/ml) used to induce p75NTR signaling in such studies may lead to the activation of signaling cascades which does not occur when p75NTR is activated to a lesser extent, as would be expected in the case of low levels of endogenous BDNF secreted in culture. Therefore, it is premature to exclude p75NTR-mediated signaling as a component of the apoptotic pathway occurring during NGF deprivation in sympathetic neurons, although the events mediated by p75NTR remain undefined under such conditions.

1.6 Sympathetic neurons as a model for studying NGF-dependent signaling

Sympathetic neurons were one of the first type of neurons demonstrated to be dependent on NGF for their survival *in vivo* (Cohen, 1960; Levi-Montalcini and Booker, 1960a). Early during development, populations of sympathetic neurons forming the sympathetic ganglia extend axons towards their target organs, often in paths that are adjacent to the blood vasculature (Rubin, 1985). Once sympathetic axons approach their target, they begin to become dependent on NGF, first for the axon growth required for the initial innervation of its targets (Glebova and Ginty, 2004), followed by a critical dependence on NGF for their survival. Sympathetic neurons become dependent on NGF for their survival between embryonic day 17.5 and post-natal day 10 (Coughlin and Collins, 1985; Kessler and Black, 1980). Transcripts for the NGF receptor, TrkA, are detected as early as embryonic day 13.5/14.5 and remain through to adulthood, indicating that this receptor is available to mediate NGF signaling during the period of NGF-dependent survival (Fagan et al., 1996). The requirement of both NGF and its receptor, TrkA, for the survival of sympathetic neurons has been verified in knockout mice; deletion of either NGF or TrkA results in almost complete loss (>95%) of sympathetic neurons in the SCG during development (Crowley et al., 1994; Smeyne et al., 1994). However, there is evidence that another neurotrophin may also regulate the survival of sympathetic neurons during development. Targeted disruption of the neurotrophin gene for NT-3 resulted in the loss of 50% of sympathetic neurons in the SCG during the developmental period when these neurons were also dependent on NGF (Francis et al., 1999; Wyatt et al., 1997). Disruption of both NGF and NT-3 in mice did not result in greater sympathetic neuron loss than in mice where only NGF had been disrupted, indicating that NGF and NT-3 control the survival of the same neurons (Francis et al., 1999). Although NT-3 is the preferential ligand for the neurotrophin receptor, TrkC, disruption of the TrkC gene in mice did not mimic the effects of the NT-3 null mice with respect to sympathetic neuron loss (Fagan et al., 1996; Tessarollo et al., 1997). Thus, it is thought that NT-3 functions by acting through TrkA instead. Targets of sympathetic neuron innervation express NT-3 (Francis et al., 1999), suggesting that NT-3 promotes survival by acting at axon terminals. However, there is a report that provides evidence that NT-3 supplied to axons is unable to support sympathetic neuron survival (Kuruvilla et al., 2004). The inability of NT-3 to support survival from axons has been attributed to the observation that although NT-3 binds to TrkA, unlike NGF bound to TrkA, it cannot undergo internalization, an event that is proposed to be required for survival mediated by neurotrophins signaling at axons. Since NT-3 is also secreted by intermediate targets along the path of axon growth of sympathetic neurons (Francis et al., 1999) and NT-3 is able to promote axon extension through TrkA (Belliveau et al., 1997), an alternative hypothesis is that NT-3 supports survival by mediating axon extension required to reach NGF-supplying targets. Consistent with this hypothesis, sympathetic neurons of NT-3 null mice fail to innervate their targets (Francis et al., 1999; Kuruvilla et al., 2004; Wyatt et al., 1997). However, in contrast to the report of Kuruvilla and co-workers (Kuruvilla et al., 2004), our laboratory has obtained data that NT-3 supplied to axons is internalized and does support the survival of sympathetic neurons (K.L and R.B.C, unpublished observations). Therefore, it is possible that *in vivo* both NGF and NT-3 mediate the target dependent survival of sympathetic neurons by activating the TrkA receptor and that NT-3 has an additional early function in mediating axon growth towards targets. Thus, although sympathetic neurons are a good neuronal model for studying NGF-mediated survival signaling, we must keep an open mind that other neurotrophins may also contribute to this process *in vivo*.

Sympathetic neurons can be isolated from the superior cervical ganglia of neonatal rats (post-natal day 0-2) and grown in dissociated cultures (Mains and Patterson, 1973). These cultured neurons are completely dependent on NGF for their survival for at least the first 10 days in culture. However, after 20 days in culture there is survival of up to 50% of neurons when NGF is removed, indicating a decrease in NGF dependency over time (Chun and Patterson, 1977a; 1977b). Therefore, young cultures of sympathetic neurons are a suitable system for studying the process of NGF-dependent survival. In addition, sympathetic neurons in culture extend long axons in the presence of NGF making them amenable for use in the compartmented culture system (Campenot, 1977).

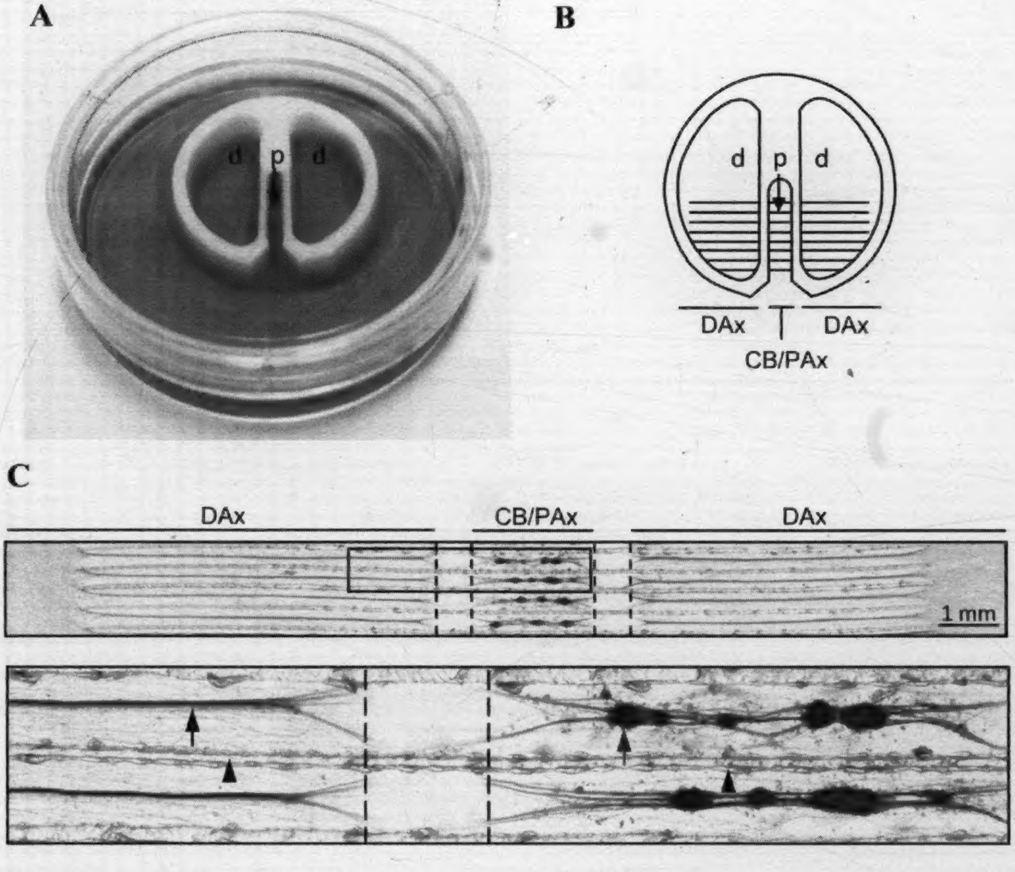
1.7 The compartmented culture system

Since NGF has been found to be secreted from the target cells innervated by sympathetic neurons *in vivo*, the secreted NGF will be concentrated at the axon terminals of these neurons and not at the distantly located cell bodies. The study of how NGF binding to axon terminals produces signals that are then retrogradely communicated, often over long distances, to the neuronal cell bodies in order to mediate survival is a major field of research. To study NGF signaling in a more physiologically relevant manner, a system should be used where NGF can be applied more specifically to axons and not the cell bodies, and allow for the ability to monitor how NGF signaling initiated in the axons leads to signaling changes in the cell bodies. The compartmented culture system is a suitable system for carrying out such NGF studies.

Established in 1977, the compartmented culture system (Figure 1-3) allows the cell bodies and proximal axons of neurons to be exposed to a separate fluid environment from their distal axons and axon terminals (Campenot, 1977). The separation of the fluid environments allows the researcher to differentially manipulate the culture conditions for the cell bodies and proximal axons, and the distal axons of the same neuron. In this system, NGF can be applied specifically to axons and axon terminals, thereby more closely approximating *in vivo* conditions. Thus, the compartmented culture serves as a valuable model for studying the mechanisms by which NGF applied to axons generates signals that travel to the cell bodies to regulate survival.

The compartmented culture is composed of a Teflon divider that is seated onto a collagen-coated tissue culture dish with silicone grease (Figure 1-3A). The divider (white) partitions the culture dish into a proximal compartment (p), and left and right distal compartments (d). Sympathetic neurons isolated from the sympathetic ganglia of post-natal (0-2 day) rats are dissociated and plated in the proximal compartment of the divider. Axons extend from the neurons within the proximal compartment and the direction of their growth is guided left and right by a set of parallel scratches made in the collagen prior to the placement of the divider (see Figure 1-3B and 1-3C). As the axons continue to extend, they pass under the divider partitions into the left and right distal compartments and continue to extend within these compartments. The resulting compartmented culture contains cell bodies and proximal axons (CB/PAx) in the proximal compartment, and distal axons (DAx) in the distal compartment. The distal axons consist of the axons that have extended from the proximal compartment. During the first 5-7 days in culture, the proximal compartment is supplied with a low

Figure 1-3. The compartmented culture system. A) A photograph of a 3-compartmented culture. A Teflon divider is seated with silicone grease onto the surface of a 35 mm collagen-coated tissue culture dish. This allows the separation of the fluids between the distal compartments (d) and proximal compartment (p) created by the divider. B) Schematic diagram of the 3-compartmented culture. The diagram shows the Teflon divider overlaid on top of tracks of collagen (horizontal lines) which are formed by making a series of scratches in the collagen coated dish. Dissociated neurons are plated in the proximal compartment (p). Plated neurons extend axons out from their cell bodies, and the direction of axon extension is channeled by the scratches in the left and right direction. The axons continue to extend under the barriers and into the distal compartments (d) where they continue to grow within this compartment. In the resulting 3-compartmented culture, the cell bodies and proximal axons (CB/PAx) of the neurons are contained in the proximal compartment, and the distal axons (DAx) which have extended out from the proximal compartment are contained in the distal compartments. C) Photomicrograph of 7-day-old compartmented culture neurons immunostained for tubulin. The top panel displays neurons in 4 out of a total of 20 tracks in a compartmented culture. The borders of the grease barriers which partition the compartments are indicated by dotted lines. The areas under the grease barriers are unstained. Note that the majority of the axon material is located in the distal compartments. The lower panel is a magnified image of the area boxed in the upper panel. The blue arrow points to a grouping of neuron cell bodies. A collagen scratch which guides the direction of axon growth is identified by arrowheads. The black arrow identifies an axon cable formed by many axons within a track in the distal axon compartment. There are also axons within each track that surround the main axon cable. There are relatively few peripheral axons in the compartment containing the cell bodies and proximal axons.


A
d
p
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B
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DAx
DAx
CB/PAx
C
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CB/PAx
DAx
1 mm

concentration of NGF (10 ng/ml) that supports survival and axon growth but not extensive axon branching (Campenot, 1987). The distal compartments are supplied with 50 ng/ml NGF, which supports axon growth and extensive axon branching. This selects for a culture in which the majority of the axon material of the neurons is contained in the distal compartments. A representative image of neurons from a 1-week-old compartmented culture is shown in Figure 1-3C. To visualize the neuron morphology the neurons have been immunostained for tubulin. Note how in the cell bodies/proximal axon compartment there are groupings of neuronal cell bodies (blue arrow points to one grouping) and axons that extend mainly left and right since they cannot extend across the collagen scratches (arrowheads). In the distal axon compartment, there are no cell bodies and many axons, some of which have coalesced into a cable (black arrow) as they extend along the collagen between the scratches. There are also many axons that are not part of the cable which also fill the tracks. By visual inspection, there is much more axonal material in the compartments containing the distal axons relative to the compartment containing the cell bodies and proximal axons. However, the compartment containing the cell bodies and proximal axons still contain all the axons that initially extend from the cell bodies prior to crossing into the distal compartments. It should also be noted that the amount of axon material within the proximal compartment can be increased by modifying the conditions of the culture, for example by increasing the concentration of NGF within the proximal compartment or impairing the ability of the axons to extend between the compartments. The neurons shown in Figure 1-3C are representative of the cultures used in the Campenot laboratory. Other laboratories also use the compartmented culture system, but these cultures are not necessarily similar to the cultures used by the

Campenot laboratory in terms of the relative amounts of axons in the compartment containing the distal axons compared to the compartment containing the cell bodies and proximal axons. Therefore, there must be a careful assessment of the compartmented culture used in each study in order to properly interpret the data obtained.

The compartmented culture system has many features that make it valuable for studying NGF-dependent retrograde signaling. For example, membrane permeable inhibitors can be applied exclusively to the distal axons or the cell bodies/proximal axons. This technique allows for the investigation of the spatial requirements of signaling within the neuron, to determine what signaling events must occur in the distal axons and cell bodies/proximal axons respectively to regulate survival. The compartmented culture system is also useful for detecting the movement of molecules such as NGF between compartments as they travel from the distal axons to the cell bodies/proximal axons and *vice versa*. Another very useful feature of the system is the ability to harvest the material of distal axons and cell bodies and proximal compartments separately for biochemical analysis and comparison. Thus, the compartmented culture system can serve as a valuable tool for elucidating the spatial organization of signaling in sympathetic neurons that regulate survival in response to NGF supplied to their distal axons.

1.8 Origins of the neurotrophic factor hypothesis

The neurotrophic factor hypothesis is a concept that is used to account for the widespread loss of neurons during development. The initial formation of the hypothesis can be traced back to a study by Hamburger and Levi-Montalcini in 1949 (Hamburger and Levi-Montalcini, 1949). In their examination of the spinal ganglia of normal developing chick embryos, they made the observation that a large number of the neurons in the ganglia were degenerating shortly after their axons reached their intended targets of innervation. In conjunction with this, they also found that removing the innervation targets increased the occurrence of degenerating cells. From these data they proposed a groundbreaking concept that during development more neurons sent axons to a shared target than the target could support and that excess neurons degenerated because the target failed to provide them with the "conditions" necessary for survival.

The identity of such survival "conditions" were a mystery at the time but would be discovered through the investigation of a diffusible factor with growth-promoting properties (Levi-Montalcini, 1952; 1954; Levi-Montalcini and Hamburger, 1951; 1953) that was eventually isolated from multiple sources (Cohen, 1954; 1960; Cohen and Levi-Montalcini, 1956). This diffusible factor was nerve growth factor (NGF) and was the first neurotrophic factor to be identified. Once NGF was isolated, several key experiments were performed which indicated that in addition to promoting axon growth, NGF also had an ability to promote survival. An anti-serum against NGF was prepared, and injection of this anti-serum into newborn rats destroyed 90-95% of the sympathetic neuron population (Cohen, 1960; Levi-Montalcini and Booker, 1960a). Conversely, injection of the isolated NGF into newborn mice induced a two-fold increase in the number sympathetic neurons observed in the superior cervical ganglia (Levi-Montalcini and Booker, 1960b). When the NGF and anti-serum injections were performed in adult mice, the effects were less severe compared to the effects seen in newborn mice (Cohen, 1960; Levi-Montalcini and Booker, 1960a), which suggested that the NGF was more critical for promoting the survival of sympathetic neurons during development.

Other experiments would provide clues to how NGF regulated survival *in vivo*. It was found that severing the axons of sympathetic neurons in the superior cervical ganglion either chemically (Angeletti and Levi-Montalcini, 1970) or surgically (Hendry, 1975) resulted in the death of the treated cells, and addition of exogenous NGF to the severed axons prevented this death (Aloe et al., 1975; Hendry, 1975). These results suggested that severing the axons prevented them from receiving a source of survival support and that resupplying NGF restored this support. It was found that target cells innervated by sympathetic neurons expressed and secreted NGF in very small quantities (Heumann et al., 1984; Korsching and Thoenen, 1983). Together these results painted a picture that would aid the development of the "neurotrophic factor hypothesis" (Levi-Montalcini and Angeletti, 1968; Purves, 1988), which proposes that a group of neurons that have extended their axons towards a shared target must compete with each other for a limited supply of neurotrophic factor secreted by that target; neurons that successfully obtain enough neurotrophic factor survive, while those that fail to receive sufficient support die.

1.9 Origins of the NGF signaling field

The first molecular hypothesis for how a neurotrophin such as NGF supported survival was proposed by Hendry in 1973 when he hypothesized that NGF could be retrogradely transported from terminals (Hendry and Iversen, 1973), "providing the cell body with a means of sampling the external environment around its axon terminals, thereby enabling the neurone to respond to trophic factors in this environment"(Hendry et al., 1974). He and others demonstrated that [^{125}I]-labeled NGF injected into sympathetic and sensory innervation targets resulted in the preferential accumulation of the labeled

NGF in the neuronal cell bodies of the innervating neurons, suggesting that the labeled NGF in the cell bodies was derived from its retrograde transport from axon terminals (Hendry et al., 1974; Stoeckel et al., 1975). Later studies provided evidence that endogenous NGF was also retrogradely transported *in vivo* (Korsching and Thoenen, 1983; Palmatier et al., 1984). To explain how NGF was being taken up and retrogradely transported, it was proposed that an NGF-specific receptor that had begun to be characterized, might mediate these processes (Hendry, 1977; Thoenen and Barde, 1980). The proposed role of an NGF-specific receptor in mediating signals was supported by the finding that introduction of NGF directly into the cytoplasm of PC12 cells did not support fibre outgrowth, a measure of NGF biological activity (Heumann et al., 1981), which indicated that NGF itself could not function as the retrograde signal and that the NGF probably required its receptor or signaling produced by its receptor to carry the retrograde message. Two receptors of NGF were cloned (Johnson et al., 1986; Klein et al., 1991) and one of them, Tropomyosin-related kinase A (TrkA), was phosphorylated and had tyrosine kinase activity in response to NGF (Kaplan et al., 1991b), suggesting that TrkA could act as a signaling mediator for NGF. The requirement of both NGF and TrkA for neuron survival *in vivo* was confirmed when it was shown that targeted disruption of either of these genes in mice resulted in the selective loss of NGF-dependent sensory and sympathetic neurons (Crowley et al., 1994; Smeyne et al., 1994). However, it was still unknown how NGF acting on TrkA at axon terminals could transmit signals to the cell bodies *in vivo*. An answer was provided when it was found that the phosphorylated TrkA receptor was retrogradely transported. In this experiment, the rat sciatic nerve, which contained axons of NGF-dependent sensory neurons, was ligated to block transport at the ligated points. Under these conditions, phosphorylated TrkA was shown to accumulate on the distal side of the ligature, indicative of retrograde transport of TrkA. In addition, both the transport and phosphorylation of TrkA were increased by the exogenous application of NGF at the innervation target of these neurons (Ehlers et al., 1995). The finding that NGF stimulated the retrograde transport of phosphorylated TrkA would provide the basis for the initial mechanisms to explain how neurotrophins, such as NGF, bind to axon terminals and generate retrograde signals that travel to the cell bodies to mediate survival.

1.10 Mechanisms of NGF retrograde signaling

What are the qualities of an NGF-dependent retrograde signal that can regulate survival? First and foremost, it should be initiated at sites where the NGF is available to bind to the neuron *i.e.* its axon terminals. Second, the signal should have the ability to be transmitted to the cell bodies and should be somewhat resistant to deactivation so that an appreciable amount of the signal arrives at the cell bodies. The signal once transmitted should induce further signaling at the cell bodies that regulates survival.

1.10.1 The signaling endosome hypothesis for NGF retrograde survival signaling

Based on the previous observations that NGF and phosphorylated TrkA were retrogradely transported (Ehlers et al., 1995; Hendry et al., 1974; Korsching and Thoenen, 1983; Palmatier et al., 1984; Stoeckel et al., 1975), a hypothesis was put forth which met the requirements for an NGF-dependent retrograde signal that could promote survival. This hypothesis, commonly referred to as the "signaling endosome hypothesis" (Beattie et al., 1996; Hendry, 1977; Howe and Mobley, 2004), proposes that NGF secreted from its target binds to its receptor, TrkA, at axon terminals and activates TrkA which leads to the internalization of both TrkA and the NGF bound to it. These NGF-

TrkA complexes are subsequently trafficked into membrane-bound organelles, termed signaling endosomes. These signaling endosomes are then retrogradely transported to the cell bodies where the phosphorylated TrkA is suitably localized to initiate downstream signaling necessary for evoking the changes in gene expression and cellular activities required for survival. An attractive feature of this hypothesis is that a retrograde carrier composed of an endosome containing TrkA in its membrane and NGF trapped in its lumen would provide stability to the signal as it travels down the axon since the trapped NGF would be continually available to maintain TrkA activation.

Since its conception, many studies have been focused on testing different aspects of this hypothesis. The hypothesis has several requirements that must be met in order to be validated: 1) NGF and activated TrkA must be internalized together in axons, 2) NGF and activated TrkA must both be retrogradely transported together, in endosomes, to the cell bodies in order to produce survival signaling and 3) once the endosomes containing NGF and activated TrkA arrive at the cell bodies, the activated TrkA is responsible for initiating the downstream signaling that supports survival.

1.10.2 Evidence for internalization of NGF and TrkA

With respect to internalization, NGF and TrkA internalization have been demonstrated in PC12 cells (Grimes et al., 1996) and at the axons of sympathetic neurons in compartmented culture (Claude et al., 1982; Kuruvilla et al., 2004). Currently there is evidence for two different mechanisms of internalization of NGF and TrkA in neurons: clathrin-dependent endocytosis (Howe et al., 2001) and macropinocytosis (Valdez et al., 2005). In PC12 cells it was shown that in isolated fractions containing clathrin-coated vesicles that some of the NGF could be chemically crosslinked to TrkA in these fractions

(Grimes et al., 1996), which suggests that at least in some vesicles NGF and TrkA were internalized together.

1.10.3 Evidence for NGF and TrkA in retrogradely transported endosomes

In order for the signaling hypothesis to be validated it must be confirmed that both NGF and TrkA are in the same endosome and that they are retrogradely transported together to the cell bodies. Following clathrin-mediated endocytosis, the vesicles fuse with organelles called early endosomes. From this point, the vesicular components are recycled to the plasma membrane, degraded, or remain associated with the endosomes to form signaling organelles (reviewed by Gruenberg, 2001; von Zastrow and Sorkin, 2007). Evidence that NGF and TrkA may be packaged into endosomes comes from a study using a preparation of excised rat sciatic nerve (Delcroix et al., 2003). Retrogradely accumulating material collected from the nerve was fractionated to yield a preparation containing markers for early endosomes, phosphorylated TrkA, and NGF. These data were interpreted as evidence that in axons, NGF and TrkA were being retrogradely transported in early endosomes (Delcroix et al., 2003). However, it should be noted that it cannot be ascertained from the data whether NGF and TrkA are always transported together in endosomes or whether a fraction of the activated TrkA is in endosomes that do not contain NGF. Of interest is the additional observation made in this study that several of the signaling molecules downstream of TrkA activation (Gab2, Rap1, B-Raf, MEK1/2, Erk1/2, p38, PI3K) were also found in the retrogradely moving endosomal fractions containing NGF and TrkA. Several of these proteins such as MEK1/2, Erk1/2, and p38 were phosphorylated, suggesting that they were activated. Once again, it could not be determined in this study if these molecules were being retrogradely carried by an association with TrkA or if they were retrogradely transported without TrkA possibly in association with non-TrkA endosomes. The retrograde transport of some of these signaling molecules (Raf, PI3K, MEK, Erk1/2) in the rat sciatic nerve was also observed in a previous study (Johanson et al., 1995).

Another study that provides evidence that retrogradely transported NGF is located in endosomes employs a technique whereby NGF is tagged to a fluorescent nanoparticle called a quantum dot. The quantum dots, each presumably bound to a single NGF dimer, were used to track the movement of NGF within axons of sensory neurons in culture (Cui et al., 2007). Additional observations provided by the study indicated that the quantum dot-NGF molecules were mostly contained in uncoated single lumen vesicles, 50-150 nm in diameter, consistent with previous published diameters for NGF-containing early endosomes isolated from sciatic nerve (Delcroix et al., 2003). The authors also estimated that the majority of quantum dot-containing endosomes possessed only one quantum dot. This was interpreted by the authors as evidence that the single NGF dimer associated with a given quantum dot was, through binding to a single TrkA dimer, capable of stimulating the internalization of the molecule into an endosome that could function as a retrograde signal. However, since the authors have no data showing that the endosomes containing quantum dot-NGF described in their study can actually mediate biological responses after their retrograde transport, it seems premature to make such a claim.

Although there is evidence to suggest that retrogradely transported NGF and TrkA are contained in early endosomes, others have described the appearance of NGF and TrkA in other membranous structures called multivesicular bodies (MVB). These multivesicular bodies consist of vesicles that have been enclosed within a second membrane and are structures normally associated with the endosome-mediated degradation pathway (reviewed by Gruenberg, 2001). In hippocampal neurons, MVBs were retrogradely transported to the cell bodies implicating them as signal carriers rather than mediators of protein degradation in the axons (Parton et al., 1992). In sympathetic neurons, MVBs containing NGF have been observed in both the axons and the cell bodies (Claude et al., 1982; Sandow et al., 2000). In the rat sciatic nerve, phosphorylated Trks were found associated with MVBs, and when the nerve was ligated, these MVBs accumulated distal to the ligature suggesting that they were being retrogradely transported (Bhattacharyya et al., 2002). However, this study also observed phosphorylated Trk associated with retrogradely transported vesicles that were not MVBs, indicating that multiple types of signaling vesicles could potentially carry activated Trk to the cell bodies. One issue regarding MVBs is how they would be able to facilitate signaling once they reached the cell bodies. Since NGF and TrkA containing vesicles would be enclosed within a second membrane it would limit the access of the intracellular domain of TrkA to interact with downstream effectors in the cytosol. Therefore, it is difficult to see how NGF and TrkA retrogradely transported in MVBs to the cell bodies could function as mediators of survival signaling unless the MVB outer membrane could be removed or the internal vesicles could fuse with the outer membrane.

1.10.4 Evidence that NGF and TrkA are retrogradely transported to the cell body

Although there is evidence that NGF and TrkA are retrogradely transported in membranous organelles such as endosomes or MVBs, it still has to be demonstrated that the NGF and TrkA are not degraded or redistributed to axon membranes during transport and instead are delivered, intact, to the cell body. Studies using NGF radio-labeled with

^{125}Iodine have demonstrated that the NGF found in the cell bodies after retrograde transport from axon terminals of sympathetic neurons *in vivo* was mostly intact (Hendry et al., 1974; Stockel et al., 1974; 1976). Experiments performed in compartmented cultures of sympathetic neurons provided evidence that [^{125}I]-labeled NGF is not degraded or released from the axon during retrograde transport (Ure and Campenot, 1997). Studies demonstrating the retrograde transport of TrkA to the cell bodies have been much less definitive. Although retrograde transport of phosphorylated TrkA within the axon was demonstrated by Ehlers et al. (1995), arrival of the phosphorylated TrkA at the cell bodies was not confirmed in that study. Several studies subsequently showed that application of NGF to the distal axons of compartmented cultures of sympathetic or sensory neurons resulted in an increase in the phosphorylation of TrkA in the cell bodies/proximal axons (Kuruvilla et al., 2000; MacInnis, 2003; Riccio et al., 1997; Senger and Campenot, 1997; Wu et al., 2007; Ye et al., 2003). However, there is no verification in these studies that the phosphorylated TrkA was derived from its retrograde transport from the distal axons. One study that attempted to demonstrate retrograde transport of TrkA to the cell bodies involved experiments in compartmented cultures of sympathetic neurons where the surface proteins in the distal axons were biotinylated prior to the application of NGF (Tsui-Pierchala and Ginty, 1999). Purification of biotinylated proteins from lysates of cell bodies/proximal axons with streptavidin after 2 h of NGF treatment at the distal axons pulled down a biotinylated, tyrosine-phosphorylated protein visualized by immunoblot with the same apparent molecular weight as TrkA suggesting that the phosphorylated TrkA was derived from its retrograde transport from the distal axons. A similar experiment in the same study, carried out with [^{125}I]-labeled NGF applied to the distal axons, demonstrated that precipitation of biotin from the cell bodies/proximal axons also pulled down [^{125}I]-labeled NGF suggesting that biotinylated, phosphorylated TrkA and NGF were retrogradely transported together. However, in this study it was not verified if the retrogradely transported TrkA localized to the cell bodies specifically, since the samples used for precipitation contained both cell bodies and proximal axon material. Another study attempted to demonstrate retrograde transport of Trk to the cell bodies by transfecting sensory neurons in compartmented cultures with TrkB-GFP fusion proteins and monitoring for fluorescence recovery in the cell bodies after photobleaching (Heerssen et al., 2004). Stimulation of distal axons with BDNF, the ligand for TrkB, resulted in increased fluorescence in the cell bodies as measured over a period of 20 minutes compared to non-stimulated controls, which could not be accounted for by *de novo* translation of the TrkB-GFP since cycloheximide did not block the increases in cell body fluorescence. Similar experiments performed with GFP fused to kinase dead TrkB or a truncated version of TrkB missing all but 12 amino acids of its intracellular domain did not produce BDNF-induced changes in cell body fluorescence after photobleaching during the period measured, suggesting that TrkB receptors required activation for retrograde transport. These results suggest that TrkB can undergo retrograde transport to the cell bodies in response to BDNF, and it is plausible that other Trk receptors such as TrkA may also be retrogradely transported to the cell bodies upon neurotrophin stimulation.

1.10.5 Determining if the mechanism predicted by the "signaling endosome hypothesis" is required for NGF retrograde survival

Given that NGF and phosphorylated TrkA are capable of being internalized and retrogradely transported in endosomes to the cell bodies as required by the "signaling endosome hypothesis", what is the evidence that once delivered they function to promote survival? Moreover, it can be asked whether endosomes containing NGF and phosphorylated TrkA are the only retrograde signals initiated by NGF at axons that are capable of supporting survival, or alternatively if NGF at distal axons initiates multiple retrograde signals that contribute to survival. Studies that have tested the requirement of endosomes containing NGF bound to phosphorylated TrkA for survival have employed various techniques to block NGF and TrkA internalization, retrograde transport, and activity at the cell bodies, then assayed the effects of these treatments on both survival signaling and, more importantly, survival.

1.10.6 Requirement of internalization for NGF retrograde survival

As stated previously, two modes of NGF and TrkA internalization in neurons have been described: clathrin-dependent endocytosis and macropinocytosis. NGF and TrkA internalization is required in order to package these molecules together into membranous organelles for retrograde transport to the cell bodies. In support of this, blocking clathrin-dependent endocytosis in sympathetic neurons by transfecting them with a dominant-negative dynamin mutant decreased the amount of [^{125}I]-NGF accumulating in the cell bodies/proximal axons in compartmented cultures of neurons supplied with [^{125}I]-NGF on their distal axons (Ye et al., 2003). Several studies have tested the requirement for internalization of NGF to mediate survival. Inhibition of either clathrin-dependent endocytosis by transfection of dominant negative dynamin (Ye et al., 2003) or macropinocytosis by transfection of dominant-negative pincher (Valdez et al., 2005) prevented NGF applied to axons of sympathetic neurons in compartmented culture from supporting survival. In compartmented cultures of sensory neurons, pharmacological inhibition of clathrin-dependent endocytosis in distal axons with monodansylcadaverine also blocked the ability of neurotrophins (BDNF plus NGF) applied to distal axons to mediate survival (Heerssen et al., 2004). The results of these studies were interpreted by the authors as confirmation that internalization of neurotrophins and their receptors was required to produce retrograde survival signals and therefore that signaling endosomes containing NGF and TrkA were the major retrograde signals contributing to NGF-mediated survival. Something that must be considered, however, is that if NGF produces other retrograde survival signals that require internalization of membranes for their retrograde transport (for example, to provide retrogradely transported vesicles to which signaling molecules can tether), then the general blockage of the entire internalization mechanism may inhibit their retrograde transmission to the cell bodies as well. Therefore, these studies cannot be used to verify that internalization of NGF and TrkA, specifically, are required for the transmission of retrograde survival signals.

1.10.7 Requirement of dynein-dependent retrograde transport for NGF retrograde survival

There is evidence to suggest that NGF and TrkA retrograde transport is mediated by motor proteins travelling along microtubules, and some studies have manipulated the transport system to test if it is required for NGF-dependent survival. Evidence that NGF retrograde transport requires microtubules was first provided by studies demonstrating that destabilizing microtubules with colchicine blocked retrograde accumulation of NGF in the cell bodies both *in vivo* (Hendry et al., 1974) and in cultured sympathetic neurons (Claude et al., 1982). The finding that TrkA is able to directly associate with the light chain of dynein *in vitro* (Yano et al., 2001) suggested that endosomes containing TrkA could be transported through an association of TrkA with dynein motors moving retrogradely along microtubules. The requirement for dynein-based transport for mediating neurotrophin retrograde survival signals was tested in sensory neurons, and it was found that interfering with dynein's association with cargo and microtubules blocked the ability of neurotrophins applied to axons to 1) be retrogradely transported to the cell bodies, 2) induce the retrograde phosphorylation of Trk receptors in the cell bodies and 3) support the survival of these neurons (Heerssen et al., 2004; Wu et al., 2007). Although the data from these studies were presented in support of retrograde transport of signaling endosomes carrying NGF and TrkA as the main retrograde signal mediating NGF-dependent survival, these studies are subject to the same issue as internalization experiments in that these studies cannot exclude the possibility that other retrograde survival signals produced by NGF might also require dynactin-dependent, dynein-based retrograde transport for their transmission to the cell bodies.

1.10.8 Experiments investigating the requirement of NGF retrograde transport for the production of downstream signaling events in the cell bodies

In contrast to the studies discussed above, there have been more specific methods for assessing the requirements for NGF internalization and retrograde transport for survival signaling and survival support. One method that has been used to specifically block NGF internalization and retrograde transport is to crosslink the NGF to 1 μm diameter beads that are too large to be endocytosed. In compartmented cultures of sympathetic neurons it was demonstrated that application of soluble NGF to the distal axons led to the phosphorylation of CREB in the nucleus of the cell bodies within 20 minutes, whereas application of NGF-bound beads to the distal axons did not induce CREB phosphorylation within the same time period (Riccio et al., 1997). Since CREB activation is implicated in NGF-dependent survival signaling, the results suggested that the NGF-bound beads failed to induce CREB phosphorylation because NGF internalization and retrograde transport were required for the activation of downstream survival signals, such as CREB, at the cell bodies.

In the same year, another study in compartmented cultures of sympathetic neurons provided evidence that retrograde transport of NGF was not required for inducing downstream survival signaling in the cell bodies/proximal axons. In this study, NGF application to the distal axons produced the phosphorylation of TrkA in the cell bodies/proximal axons within 1 minute and other proteins became tyrosine phosphorylated within 5-10 minutes (Senger and Campenot, 1997). However, when the retrograde transport of [^{125}I]-NGF from the distal axons was measured in this study, it was found that the [^{125}I]-NGF could not be detected in the cell bodies/proximal axons for at least 30 minutes. These results suggested that NGF applied to the distal axons could produce a rapid retrograde signal that phosphorylated TrkA and other molecules in the cell bodies/proximal axons by a mechanism not requiring the retrograde transport of NGF to the cell bodies. By extension, it would also be expected that endosomes containing NGF and phosphorylated TrkA could not account for the results observed.

The observation that NGF retrograde transport could not be detected in compartments containing the cell bodies/proximal axons for 30 minutes following NGF stimulation raises the question of how the CREB phosphorylation detected after only 20 minutes of NGF stimulation in the Riccio et al. (1997) study could have been the result of NGF retrograde transport. Since the retrograde transport of soluble NGF was not examined in that particular study, the possibility that NGF retrograde transport could have occurred in that time period cannot be excluded. However, several studies carried out in compartmented cultures of sympathetic or sensory neurons, in agreement with the Senger and Campenot (1997) report, have observed a significant amount of lag time between the addition of NGF to the distal axons and its detection in the cell bodies. These delay times have been recorded to be about 1 h for [^{125}I]-NGF (Claude et al., 1982; Ure and Campenot, 1997) and 40 minutes for NGF tagged with fluorescent quantum dots (Cui et al., 2007). Thus, it is plausible that CREB phosphorylation in the cell bodies seen after only 20 minutes of NGF stimulation on the distal axons was mediated by a retrograde signal that did not involve the retrograde transport of NGF. However, what would account for the observation that free NGF applied to the distal axons induced nuclear CREB phosphorylation in the cell bodies but that bead-bound NGF did not, if the retrograde signal can be produced without the retrograde transport of NGF? One reason for this discrepancy may be due to the differing ability of bead-bound NGF and free NGF to induce Trk activation in this study. It is evident from immunoblots that the concentration of bead-bound NGF used in the experiment was not as effective at inducing Trk phosphorylation as the concentration of free NGF (100 ng/ml) used for comparison. Moreover, in the experiment analyzing CREB phosphorylation an even higher concentration of free NGF was used (200 ng/ml) for comparison with NGF bound to beads. Therefore, it is possible that in this experiment, in comparison to free NGF, the bead bound NGF did not produce a retrograde signal large enough to induce CREB phosphorylation in the cell bodies within the time period assayed.

It has been suggested that the [^{125}I] assay used to measure retrograde transport of NGF in Senger and Campenot (1997) may have lacked the sensitivity to detect the retrograde transport of NGF that might have been occurring within 1 minute of NGF stimulation and thus it is possible that the phosphorylated TrkA observed in the cell bodies/proximal axons may have been a result of the retrograde transport of endosomes containing NGF and phosphorylated TrkA (Ginty and Segal, 2002). Given that the fastest retrograde transport rates for NGF in compartmented cultures were estimated to be 10-20 mm/h (Ure and Campenot, 1997), the shortest time required for NGF to be transported across the 1 mm barrier separating the distal axons from the cell bodies/proximal axons is 3 minutes. Therefore, even assuming that NGF adjacent to the barrier could bind TrkA, be immediately internalized, and retrogradely transported at the maximum rate, it would still take a minimum of 3 minutes for any NGF and associated TrkA to reach the cell bodies/proximal axons. Since TrkA phosphorylation was observed within 1 minute of NGF addition to the distal axons, it is unlikely that the retrograde transport of endosomes containing NGF and TrkA could have accounted for the NGF retrograde signal observed.

What signaling mechanism could account for the rapid phosphorylation of TrkA in the cell bodies/proximal axons observed by Senger and Campenot (1997)? A hypothesis that has been put forth by the authors is that NGF binding to Trk receptors in the distal axons induces their phosphorylation that then instigates activation and phosphorylation of nearby unoccupied Trk receptors, followed by propagation of the activation of Trk receptors along the axon which spreads all the way to the cell bodies. Consistent with this hypothesis, propagation of receptor phosphorylation has been described for ErbB1/EGFR in the MCF7 cell line (Verveer et al., 2000). However, whether such a propagating signal is sustainable or contributes to neuronal survival is uncertain. Experiments presented later in this introduction provide evidence that TrkA phosphorylation in the cell bodies/proximal axons is not required for retrograde survival signaling and argue against an essential role for this type of signal in supporting neuronal survival.

1.10.9 Investigating if NGF retrograde transport is required for NGF retrograde survival

Another study subsequent to the report of Senger and Campenot (1997) supports the concept that the retrograde transport of NGF is not required to produce retrograde signals that support survival. The study employed [^{125}I]-NGF bound to beads at a concentration that stimulated the phosphorylation of TrkA to similar levels observed with concentrations of free NGF (50 ng/ml) that supported neuron survival (MacInnis and Campenot, 2002). In experiments with compartmented cultures of sympathetic neurons, application of bead-bound [^{125}I]-NGF to the distal axons supported the survival of the majority of neurons (84%) as measured at 30 h. At the end of the experiment, radioactivity counts of the media from the compartment containing the cell bodies/proximal axons which represented retrogradely transported NGF that been degraded and released, revealed that the amount of NGF retrogradely transported during the experiment was barely detectable above background. In the same experiment, several concentrations of free [^{125}I]-NGF were also assayed and revealed that cultures given 0.5 ng/ml free [^{125}I]-NGF only supported the survival of 30% of neurons in culture and yet transported 20 times more NGF than could have been transported form [^{125}I]-NGF bound to beads. These results indicate that the small amount of [^{125}I]-NGF that may have been retrogradely transported under bead-bound NGF conditions could not have mediated the survival observed and therefore is evidence supporting the concept that NGF is capable of producing retrograde signal(s) that are sufficient for supporting neuron survival via mechanism(s) that do not require the retrograde transport of NGF. The findings of the above study are supported by experiments performed in sensory neurons whose survival can be mediated by both NGF and BDNF. In compartmented cultures of sensory neurons, application of bead bound BDNF supported neuronal survival at levels similar to that achieved with free BDNF (Heerssen et al., 2004), suggesting that similar to NGF, retrograde transport of BDNF was not required to produce retrograde signals capable of supporting survival. In this study there was also evidence that the receptor for BDNF, TrkB, was internalized in response to the application of bead-bound BDNF, raising the possibility that the TrkB receptors were retrogradely transported under these conditions and could function as a retrograde survival signal. In contrast to this finding, retrograde transport of TrkA was not detected when distal axons were stimulated with NGF bound to beads (MacInnis, 2003), suggesting that Trk receptors may differ in the types of retrograde survival signals they produce.

In contrast to the reports above, experiments carried out by Ye et al. (2003) presented evidence in support of a requirement of retrogradely transported NGF for survival. Rather than blocking the retrograde transport of NGF, they attempted to neutralize the NGF once it reached the cell bodies/proximal axons with anti-NGF antibodies. To allow the anti-NGF to access the inside of NGF-containing structures such as endosomes, they delivered the anti-NGF using a carrier peptide, Pep-1, which is able to penetrate membranes. Anti-NGF, but not rabbit IgG, delivered inside the cell bodies/proximal axons of sympathetic neurons in compartmented cultures partially blocked their survival when they were supplied with NGF only on their distal axons. Since according to the "signaling endosome hypothesis", NGF functions in endosomes to maintain the activation of TrkA required for downstream signaling mediating survival, the results of the antibody experiments were interpreted as evidence that signaling endosomes carrying both NGF and TrkA function as retrograde signals that are necessary for survival. These findings appear to contradict the report by MacInnis et al. (2002) that retrograde transport of NGF is not required for retrograde signaling that supports survival. It is possible that since different techniques were used between the studies, there is some unrecognized side effect of one of the techniques that could account for the discrepancy. However, other evidence presented in the Ye et al. (2003) study also supports a role for endosomes carrying NGF and TrkA as essential retrograde survival signals. This evidence will be discussed in detail in the following Section along with a hypothesis for why conflicting reports may have been obtained by different studies.

1.10.10 Studies investigating the requirement of phosphorylated TrkA at the cell bodies for mediating NGF retrograde survival

The signaling endosome hypothesis predicts that the arrival of endosomes containing NGF and phosphorylated TrkA at the cell bodies allows for the phosphorylated TrkA to induce downstream signaling that supports survival. Therefore, if the position is taken that signaling endosomes containing NGF and phosphorylated TrkA are retrograde signals essential for survival, then inhibiting TrkA phosphorylation in the cell bodies should prevent survival. Several studies have employed a general inhibitor of Trk receptors, K252a, to test the requirements of TrkA phosphorylation for mediating NGF retrograde survival. In compartmented cultures of sympathetic neurons it was shown that when NGF was supplied to the distal axons, application of K252a to the cell bodies/proximal axons prevented the phosphorylation of TrkA, Akt, and CREB in the cell bodies/proximal axons (Kuruvilla et al., 2000; Riccio et al., 1997). In similar experiments with neurotrophin (BDNF plus NGF) stimulation of sensory neurons, application of K252a to the cell bodies/proximal axons blocked Erk5 phosphorylation in the cell bodies (Watson et al., 2001) and partially prevented survival (Heerssen et al., 2004). For sympathetic neurons, it was demonstrated by one study using compartmented cultures that when NGF was supplied to the distal axons, inhibiting TrkA in either the distal axons or the cell bodies/proximal axons with K252a prevented survival (Ye et al., 2003). This was the same study that also showed a requirement for NGF function in the cell bodies/proximal axons for survival. Taken together, the results were interpreted by the authors as being consistent with the signal predicted by the "signaling endosome hypothesis" as being a major mediator of survival.

A paper published subsequent to Ye et al. (2003) has also examined the requirements for TrkA phosphorylation in the cell bodies. Using compartmented cultures of sympathetic neurons supplied with NGF on their distal axons, MacInnis et al. (2003) found that inhibiting TrkA with K252a in the distal axons prevented survival which was expected if TrkA activity is required for the generation of retrograde survival signals.

However, it was also found in this study that inhibiting TrkA in the cell bodies/proximal axons did not significantly prevent survival, suggesting NGF retrograde survival signals do not require TrkA activity in the cell bodies for the effective induction of downstream survival signaling required for survival. In support of this, it was shown in the same study that inhibition of TrkA in the cell bodies/proximal axons did not block the ability of NGF applied to the distal axons to induce the phosphorylation of the downstream signaling molecules, Akt and CREB, in the cell bodies/proximal axons. The results presented in the study indicate that NGF applied to axons produces retrograde survival signals that do not require the phosphorylation of TrkA at the cell body to mediate their effective transmission. This raises the possibility that NGF is capable of utilizing other signaling molecules downstream of NGF and TrkA for the retrograde transmission of signals to the cell bodies that induce survival signaling.

What could account for the differences in results obtained by the studies of Ye et al. (2003) and MacInnis et al. (2003) regarding the requirement of phosphorylated TrkA at the cell bodies/proximal axons for survival? Both studies were carried out in compartmented cultures of sympathetic neurons. With respect to experimental variables, both studies supplied 10 ng/ml NGF to the distal axons, assayed similar concentrations of K252a (100 nM, MacInnis et al. also tested 200 nM and 500 nM), assessed survival at the same time point (48 h), and used the same assay to measure neuronal survival (nuclear morphology). One potential source of variation that could account for the difference in results might be the compartmented cultures themselves. Upon analyzing the image of a compartmented culture provided in Ye et al. (2003), it is observed that the majority of the axonal material within the cultures is present in the compartment containing the cell bodies. The relatively small amounts of axonal material present in the distal compartments suggest that most of the axons in the culture have failed to cross from the compartment containing the cell bodies into the adjacent distal compartments. Immunoblots provide additional information that the majority of the TrkA is present in the cellular material from the compartment containing the cell bodies and proximal axons. In comparison, in compartmented cultures used in the MacInnis et al. (2003) study, the majority of the axonal material and TrkA were found in the distal compartments and not in the compartment containing the cell bodies and proximal axons (MacInnis et al., 2003; Senger and Campenot, 1997, and Figure 1-3C). When the axon extension rates in these two cultures are compared, it is observed that the same concentration of NGF (10 ng/ml) produced extension rates of 1.3 mm/day in the cultures of MacInnis et al. (2003) but only 0.25 mm/day in the cultures of Ye et al. (2003). The diminished axon growth capacity of neurons used in Ye et al. (2003), a process that is mediated by NGF signaling, suggests that its ability to respond to NGF for other signaling processes may also be impaired or otherwise limited. One hypothesis for the different results between the studies by MacInnis et al. (2003) and Ye et al. (2003) is that the differences in the distributions of axonal material in compartmented cultures or other culture conditions may affect the signaling capabilities of neurons in response to NGF, *i.e.* the retrograde signaling mechanisms that can effectively support survival of the neurons may vary depending on the state of the compartmented culture. According to this hypothesis, the compartmented culture neurons used in the MacInnis et al. (2003) study are capable of producing retrograde survival signals that do not require the phosphorylation of TrkA in the cell bodies to support survival, whereas compartmented culture neurons used in the Ye et al. (2003) study cannot use such retrograde signals to effectively support survival. Since Ye et al. (2003) also found a requirement for NGF function in the cell bodies/proximal axons, it is possible that their compartmented culture neurons do utilize endosomes containing NGF and TrkA as a retrograde survival signal.

In addition to the conflicting results obtained by the Trk inhibition studies with K252a, another issue has arisen regarding the usefulness of K252a as a Trk inhibitor. In the MacInnis et al. (2003) study, it was found that the highest concentration of K252a used (500 nM) promoted a significant amount of survival when applied to the cell bodies/proximal axons of compartmented cultured sympathetic neurons, even when the cultures were not supplied with any NGF. The ability of high concentrations of K252a to produce pro-survival side effects complicated the interpretation of the results in this study. Thus, in light of the conflicting evidence obtained with K252a by different studies and the potential side effects associated with K252a use, the issue of whether TrkA phosphorylation in the cell bodies is required to promote survival needs to be further investigated.

1.10.11 Identification of signaling molecules downstream of NGF and TrkA that function as retrograde signals that mediate survival

In a recent study using compartmented cultures of sensory neurons it was found that CREB was locally translated in axons in response to NGF and that the retrograde transport of this newly synthesized CREB was required for the survival of these neurons (Cox et al., 2008). Thus, in sensory neurons, axon-translated CREB functions as an NGF-mediated retrograde survival signal. Whether or not this retrograde signal also operates in sympathetic neurons needs to be determined.

1.10.12 Summary of potential NGF retrograde signals mediating survival based on existing studies

After decades of research on NGF retrograde survival signaling, where does the field stand? The predictions of the "signaling endosome hypothesis" have been very useful over the years for providing a framework for exploring various aspects of NGF retrograde signals that regulate survival. There is evidence to support that endosomes carrying NGF and phosphorylated TrkA are retrogradely transported though axons as proposed by the "signaling endosome hypothesis", and it is plausible that these endosomes are capable of promoting survival. However, it is also evident from several studies that NGF produces other retrograde signals capable of effectively supporting survival that cannot be composed of endosomes containing NGF and TrkA, since these signals can still mediate survival when either retrograde transport of NGF or phosphorylation of TrkA at the cell bodies/proximal axons are inhibited. Based on these studies, it is reasonable to hypothesize that NGF produces multiple retrograde signals that promote survival. It still remains to be determined how many retrograde signals operate within neurons and whether all retrograde signals can be generated by NGF with equal efficiency under various conditions. Studies involving inhibition of phosphorylated TrkA would seem to suggest that neurons cultured under different conditions may differ in their ability to utilize certain retrograde signals to mediate survival. In regards to defining molecular components of the various NGF retrograde survival signals it is probable that some signals may be carried by signaling molecules that are activated downstream of NGF and TrkA in axons. An example of such a signal that has already been identified in sensory neurons is axon-translated CREB, but one should remain open to the possibility that other signaling molecules also function as retrograde signals.

The existing studies have made us aware that there is still much to learn about the diversity of NGF retrograde signals that regulate survival, their molecular mechanisms, and under what conditions they may operate. Continued study of NGF retrograde signals will not only provide an understanding of how neuron survival is regulated during development but also provide insight into the overall process of how axons communicate information to the cell bodies, a process that may continue to be used by neurons throughout life to respond to their environment, including deleterious conditions such as those imposed by neurotrauma and neurodegenerative diseases.

1.11 Introductory synopsis

In this thesis, two different NGF-dependent retrograde signals will be examined. The first study re-examines the issue of whether NGF retrograde signals require TrkA phosphorylation at the cell bodies/proximal axons to mediate survival. TrkA inhibitor studies have produced two conflicting reports: one that supports a requirement for TrkA phosphorylation at the cell bodies for survival (Ye et al., 2003) and one that does not (MacInnis et al., 2003). In addition, there is evidence that the Trk inhibitor used in these studies, K252a, has a non-specific effect that complicates data interpretation (MacInnis et al., 2003). The results obtained in this thesis employ an alternative TrkA inhibitor, Gö6976, to demonstrate that phosphorylated TrkA is not required in the cell bodies to promote survival. Therefore, these results define an NGF retrograde signal in sympathetic neurons that does not utilize TrkA at the cell bodies to mediate downstream signaling events yet which are fully capable of supporting neuron survival.

The second study in this thesis provides evidence for a novel NGF-dependent retrograde signaling mechanism that regulates neuron survival: an axon apoptotic signal. Apoptotic signaling in response to NGF withdrawal has been studied to some extent in sympathetic neurons; however, in contrast to the studies examining NGF survival signaling, there has been little effort to characterize where these signals occur within the neuron. The data presented in this thesis provide evidence that NGF signaling in axons promotes survival by suppressing the generation of an axon apoptotic signal. Loss of NGF signaling in axons leads to the induction of the axon apoptotic signal that is then retrogradely transmitted to the cell bodies to promote apoptotic signaling events. The axon apoptotic signal is the first evidence that axons are capable of generating a retrograde apoptotic signal that is regulated by NGF.

CHAPTER 2:

MATERIALS AND METHODS

2.1 Rat sympathetic neuron cultures

2.1.1 Preparation of culture media

The composition of the media solutions used to culture rat sympathetic neurons in all the experiments contained in this thesis are listed in Table 2-1. A detailed guide for media preparation is located in Campenot et al. (2009). Base medium is an L-15 medium formulation modified to optimize metabolism, growth, and survival of sympathetic neurons in culture (Mains and Patterson, 1973). For compartmented cultures, it was found that rat serum and *L*-ascorbic acid were detrimental to distal axon health (Campenot, 1982b) and therefore these additives were omitted from the distal compartment medium. Since non-neuronal cells were only present in the proximal compartment, the anti-mitotic agent, cytosine arabinoside, was only added to the proximal compartment medium.

Table 2-1. Culturing media

Medium	**Composition**	**Original reference**
Base medium	1.2% (w/v) L-15 powder, 18.4 µM *L*-aspartic acid, 16 µM *L*-glutamic acid, 21 µM *L*-proline, 10.2 µM *L*-cystine, 6 µM *p*-aminobenzoic acid, 9 µM β-alanine, 246 nM vitamin B12, 9 µM myo-inositol, 11.5 µM choline chloride, 35.2 µM fumaric acid, 85 nM Coenzyme A, 134 nM *D*-biotin, 39 µM lipoic acid, 68 µM imidazole, 24 µM phenol red, pH 7.35, 0.21 % $NaHCO_3$, 0.4% methylcellulose, 33 mM *D*-glucose, 2 mM *L*-glutamine, 100 U ml^{-1} penicillin, 0.01% (w/v) streptomycin, 8 µM glutathione, 22 µM 6,7-dimethyl-5,6,7,8-tetrahydropterine	modified from Hawrot and Patterson (1979)
Dissection medium	Base medium minus $NaHCO_3$ and methylcellulose	modified from Hawrot and Patterson (1979)
Proximal compartment medium	Base medium, 2.5% (v/v) rat serum, 300 µM L-ascorbic acid , 10 µM cytosine arabinoside, 10 ng/ml NGF	Campenot, 1982
Distal compartment medium	Base medium, 50 ng/ml NGF	Campenot, 1982

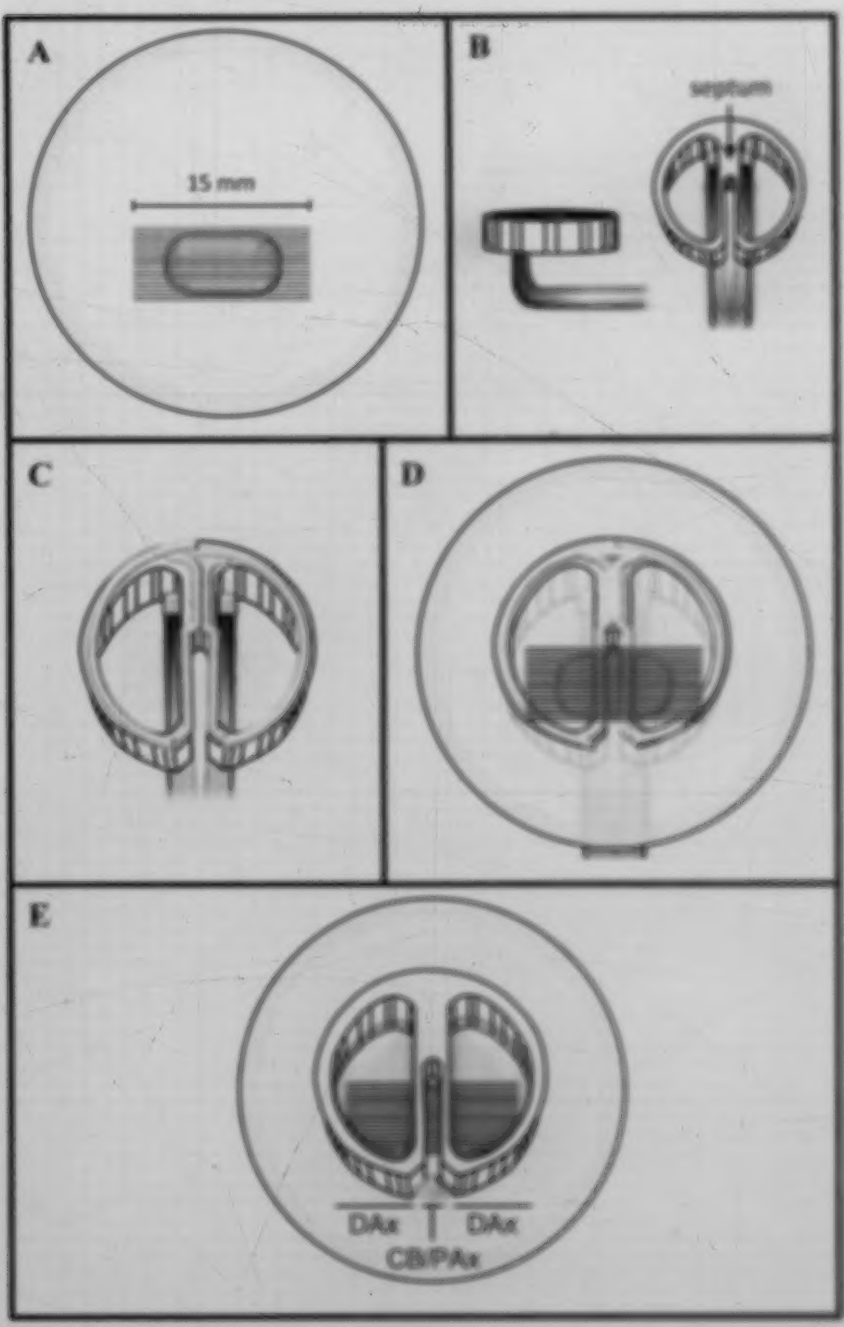
Table 2-1. continued…

Medium	Composition	Original reference
Intermediate compartment medium	Base medium, 15 ng/ml NGF	Campenot et al., 1996
Mass culture medium	Base medium, 2.5% (v/v) rat serum, 300 μM L-ascorbic acid , 10 μM cytosine arabinoside, 50 ng/ml NGF	modified from Hawrot and Patterson, 1979

2.1.2 Assembly of compartmented cultures

A recent and in-depth description for the construction of compartmented cultures is found in Campenot et al. (2009). A depiction of the assembly of standard 3-compartmented cultures is shown in Fig. 2-1. In brief, 35 mm tissue culture dishes were coated with a solution of rat tail collagen that was prepared in the lab based on the method of Hawrot and Patterson (1979) or made with a commercial source of rat tail collagen (Sigma, reconstituted as 0.1 mg/ml solution in 0.08% acetic acid). A pin rake, which is a hand-held rod containing at one of its ends a set of pins with their tips aligned in a straight row, was used to make a series of parallel scratches into the dish surface (Fig. 2-1A). A droplet of distal compartment medium was applied to the scratched region. The partitions of the compartment culture were formed by a Teflon divider (Tyler Research) that was adhered to the dish with a thin layer of silicon grease. 90° hemostatic forceps were used to clamp the Teflon divider and suspend the divider above the work surface (Figure 2-1B). Strips of silicone grease (Dow Corning) were applied to all partitions of the divider (Figure 2-1C). The prepared tissue culture dish was then inverted and seated onto the divider (Figure 2-1D). It was verified that the droplet covered the region of the partitions through which the axons were expected to extend; the liquid

Figure 2-1. Schematic of compartment culture assembly. A) A set of parallel scratches is made into the surface of a collagen coated dish with a pin rake. The scratches are approximately 15 mm in length, long enough to span the width of the divider. A droplet of distal compartment medium is laid within the scratched region as depicted, and the dish is set aside temporarily. Once the droplet has been applied, the assembly of the culture is completed within 5 min. B) A Teflon divider is clamped with a pair of 90° hemostatic forceps and positioned over the work surface. The clamped divider is shown from a side view (left) and top view (right). C) Strips of grease are applied to the divider surface in the pattern shown. Note that intersecting strips of grease contact each other or are slightly overlapping. D) The prepared dish is flipped over and placed on top of the greased divider. Light pressure is applied with a pair of forceps over the greased areas to seal the grease to the dish; however, no pressure is applied in the droplet region where the axons are expected to cross. E) The dish and attached divider are flipped over and released from the forceps. The opening of the proximal compartment containing the cell bodies/proximal axons (CB/PAx) is plugged with a small mound of grease. Two droplets of distal compartment medium are placed in each distal compartment that will contain the distal axons (DAx) of the cultured neurons.


A
15 mm
B
septum
C
D
E
DAx
DAx
CB/PAx

interface prevented the grease from completely adhering to the substratum in that area (Fig. 2-1A). Light pressure was used to seal the dish with the grease and the divider. The dish and attached divider were then inverted and released from the forceps. Any gaps in the grease seal were patched with additional grease and the proximal compartment opening was plugged with a small mound of grease (Fig. 2-1E). Two droplets of distal compartment medium were added to each distal compartment to prevent the original droplet from drying and forming a precipitate on the collagen. The completed compartmented culture was placed in the tissue culture incubator to strengthen the grease seal for a minimum of 3 h (maximum = 24 h). Prior to plating, the distal compartments were filled with distal compartment medium.

The assembly of 3-compartmented cultures (Fig. 2-2) was essentially the same as that described for 2-compartmented cultures with the exception that the scratches were lengthened to accommodate the wider divider and the intermediate compartments were filled with intermediate compartment medium prior to plating. The width dimension of each compartment in both the 2-compartmented cultures and 3-compartmented cultures is shown in Figure 2-2 for comparison.

2.1.3 Dissociation of rat sympathetic neurons

Sympathetic neurons in this thesis were obtained from the superior cervical ganglion (SCG) of 0- to 2-day old Sprague-Dawley rats (Health Sciences Laboratory Animal Services, University of Alberta). The SCGs are visualized as translucent spindle-like structures located in the neck, lying adjacent to the carotid arteries at the point where the arteries bifurcate. There are two SCGs per animal: one located on the left carotid artery and the other located on the right carotid artery. The isolated SCGs were collected

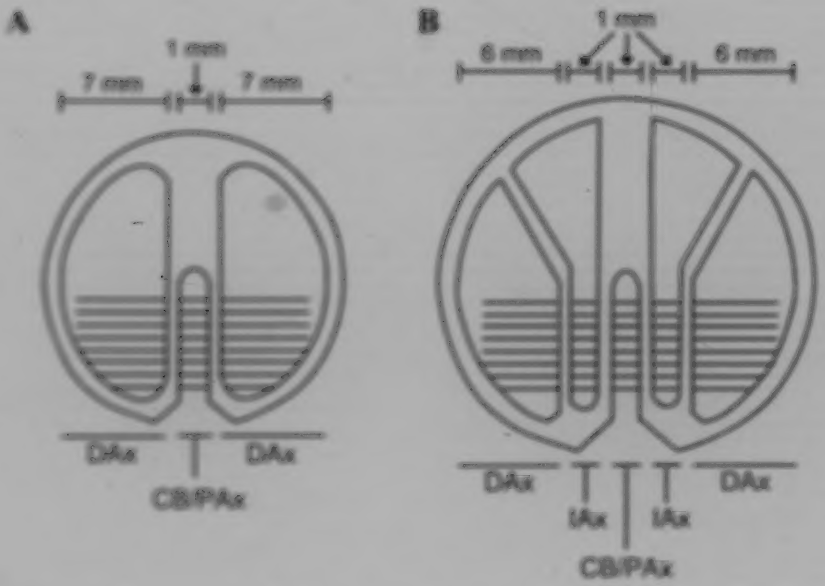

A
1 mm
7 mm
7 mm
DAx
DAx
CB/PAx
B
1 mm
6 mm
6 mm
DAx
IAx
IAx
DAx
CB/PAx

Figure 2.2. Width dimensions of 3- and 5-compartmented cultures. Schematic of dividers overlaid on a collagen surface that has been scratched (horizontal lines) to form tracks of collagen. For clarity, only 7 out of 20 tracks are drawn. A) 3-compartmented culture. B) 5-compartmented culture. CB/PAx = cell bodies/proximal axons, DAx=distal axons, IAx=intermediate axons.

in dissection medium. The SCGs were first digested with 0.1% (w/v) collagenase (Sigma) dissolved in PBS for 25 min at 37°C, then trypsin (Sigma) was added at a final concentration of 0.08 % (w/v), and the SCGs were digested for an additional 5 min at 37°C. The digestion reaction was stopped by the addition of 10% rat serum in dissection medium. The SCGs were resuspended in dissection medium and dissociated by triturating the SCGs through the opening of a p200 micropipette tip. Dissociated cells were pelleted and resuspended in the appropriate volume of proximal compartment medium in preparation for plating.

2.1.4 Plating and culture conditions for compartmented cultures

Dissociated cells were resuspended in proximal compartmented medium at a concentration of 5 ganglia per ml: each ml was sufficient to plate 12 compartmented cultures. The cell suspension was drawn up into a disposable syringe fitted with a 22 gauge needle. The cells were plated by ejecting the suspension from the syringe into the proximal compartment of the cultures until the compartment was completely filled. The plated cells were then allowed to settle onto the collagen surface overnight in the incubator. The following morning, the perimeter of the dish was filled with proximal compartmented medium until it connected with the existing plated medium. 3-compartmented cultures were incubated for 6-8 days prior to experimental treatments. During this incubation, non-neuronal cells were removed by the action of cytosine arabinoside, and the axons of the sympathetic neurons extended and crossed into the distal compartments.

For 5-compartmented cultures, extending axons crossed into an intermediate compartment prior to crossing into the distal compartment. In this thesis, the purpose of the 5-compartmented culture was to test mechanistic differences between events occurring in an intervening length of axon from those occurring in axons containing the axon terminals. To achieve the desired experimental setup, the cultures were allowed to grow for 5-6 days; at this point many axons had crossed into the distal compartments. The medium in the intermediate compartment was then replaced with base medium that lacked NGF, and proximal compartments were supplied with proximal compartmented medium also lacking NGF. The cultures were incubated for an additional 2 days, during which time the only source of NGF was in the distal compartments, and therefore neurons that had not yet projected axons into the distal compartments did not receive NGF and underwent apoptosis. Additionally, axon branches terminating in the intermediate and proximal compartments degenerated. The final result prior to experimental treatment was neurons selected to contain cell bodies in the proximal compartment, with their axons extending through the intermediate compartment and into the distal compartment where they terminated.

2.1.5 Plating and culture conditions for mass cultures

In preparation for plating, mass culture wells were coated with collagen as described for compartmented cultures. Dissociated cells were resuspended in mass culture medium. The amount of cells plated per well was dependent on the well size: 0.5 ganglia per well for 48-well plates, 0.12 ganglia per well for 96-well plates. The cell suspension was diluted in sufficient volume so that the plated medium covered the entire surface of the well for the duration of the overnight incubation. The following morning, wells were topped up with mass culture medium and incubated for 6-8 days prior to use in experiments.

2.2 Setup of experimental treatments

2.2.1 Preparation of media

For experimental treatments, the medium for proximal compartments and mass cultures consisted of base medium supplemented with 2.5% rat serum and 300 µM *L*-ascorbic acid. For intermediate and distal compartments, base medium alone was the foundation. To each medium, one of the following was added: NGF (50ng/ml unless otherwise indicated) to allow for NGF-mediated survival signaling, or 24 nM NGF function-blocking antibodies (Cedarlane) to prevent NGF signaling (Campenot et al., 1991).

2.2.2 Preparation of inhibitors and addition to media

Lyophilized inhibitors were pre-dissolved into stock solutions with the solvent recommended by the supplier (based on solubility). Prepared stocks were stored in single-use aliquots at -20°C except for colchicine and LiCl, which were freshly prepared for each experiment. Information for stock solutions of each inhibitor is listed below:

Table 2-2 Inhibitors

Inhibitor	Company	Stock [ ]	Solvent	Reference
chelerythrine	Calbiochem	5 mM	DMSO	Herbert et al. (1990)
colchicine	Sigma	5 mM	H_2O	LaBorde and Houde (1887), Bhattacharyya et al. (2008)
Gö6976	Calbiochem	500 µM	DMSO	Martiny-Baron et al. (1993)
K252a	Calbiochem	2 mM	DMSO	Kase et al. (1986)
Kenpaullone	Calbiochem	2.5 mM	DMSO	Zaharevitz et al. (1999)

Table 2-2. continued…

Inhibitor	Company	Stock [ ]	Solvent	Reference
LiCl	Sigma	150 mM	H_2O	Klein and Melton (1996)
NSC23766	Calbiochem	9.4 mM	H_2O	Gao et al. (2004)
Rottlerin	Calbiochem	1.25 mM	DMSO	Gschwendt et al. (1994)

Immediately prior to use, inhibitor stock solutions in dimethylsulfoxide (DMSO, Sigma) were diluted as necessary with additional DMSO to obtain a secondary stock solution that was 500 X the final desired concentration of the inhibitor in medium. Media solutions were warmed to 37°C prior to the addition of inhibitors. The prepared inhibitor stock solutions were added to the medium at a dilution of 1:500. The final concentration of DMSO in the medium upon addition of the inhibitor was 0.2%. In experiments where inhibitors were used, all compartments not treated with inhibitors were supplemented with 0.2% DMSO, including the compartments of compartmented cultures that did not receive any inhibitor treatment.

The water-soluble inhibitors, colchicine and NSC23766, were supplied to medium by direct addition of the stock solutions (2-9.4 µl) to achieve the desired final concentration. Equivalent volumes of double-distilled H_20 were added to the medium supplied to all compartments not receiving inhibitors.

The concentration of LiCl (15 mM) used in experiments had the potential to affect the osmolarity of the final medium depending on how it was added. To eliminate the changes in osmolarity, LiCl was prepared as a 150 mM stock solution. The osmolarity of this solution was similar to that of the L-15 medium on which our media is based. The appropriate volume of stock solution was added to media to achieve the final concentration. Media not containing LiCl were supplied with an equivalent dilution volume of a 150 mM NaCl (EMD) solution.

2.2.3 Treatments in compartmented cultures

Culture medium in the perimeter of the culture dish was removed by a Pasteur pipette connected to an aspirator. This same method was then used to remove the medium from the distal compartments. Care was taken to not disturb axon attachment during medium removal. Approximately 130 μl of treatment medium was supplied to each distal compartment. Medium in the proximal compartments was removed with a gel-loading tip fitted over the end of the pipette-aspirator setup. Approximately 80 μl of treatment medium was supplied to each proximal compartment, leaving the perimeter empty, unless the medium contained NGF, in which case, approximately 2 ml was supplied to fill both the proximal compartment and the perimeter of the dish. Treated cultures were incubated for the time indicated in the experiments. When the media needed to be changed during an experiment, the same procedure described above was followed with the exception that the medium from the distal compartments was removed by slowly drawing the solution into a p1000 micropipette. This gentler method of removal minimized the disturbance of axons that became weakly attached during NGF withdrawal. Media changed every 24 h except for Gö6976 and K252a experiments, in which media was changed every 10-14 h.

2.2.4 Treatments in mass cultures

Medium was removed from mass culture wells with a p1000 micropipette. The treatment medium was then added to each well: 300 μl for 48-well plates and 100 μl for 96-well plates. Treated cultures were incubated for the time indicated in the experiment.

2.3 Measuring neuronal survival and apoptosis in Hoescht-stained nuclei

At the end of experimental treatments, mass culture wells or the proximal compartments of compartmented cultures containing the neuronal cell bodies were rinsed once with phosphate-buffered saline (PBS) then fixed with 4% (w/v) paraformaldehyde (Fisher) in PBS for 15 min at room temperature. The fixed material was then incubated with 10 μM Hoechst 33258 (Molecular Probes) in PBS for 10-15 min until stained nuclei were visible under a microscope with a UV filter. The cultures were rinsed with PBS to remove excess staining solution. For compartmented cultures, solutions were removed from all compartments, and the Teflon divider was gently lifted from the dish. Mounting medium was applied to cover the cell surface, and a coverslip was mounted with light pressure. For mass cultures, the PBS rinse was replaced with mounting medium, and the sample was not further processed. Nuclear morphology of individual neurons was examined using a Leica DMIRE2 microscope with a UV filter (excitation filter = 360-440 nm). Round, stained, nuclei that filled a significant portion of the cell body area were scored as non-apoptotic (Fig. 2-3). Neurons that contained condensed, fragmented nuclei and neurons that lacked nuclear staining were scored as apoptotic. The presence of neurons lacking nuclei was verified under phase contrast illumination. At 48 h and 72 h time-point experiments, the majority of neurons that had undergone apoptosis lacked nuclei and had to be quantified by the presence of their cell bodies in phase contrast.

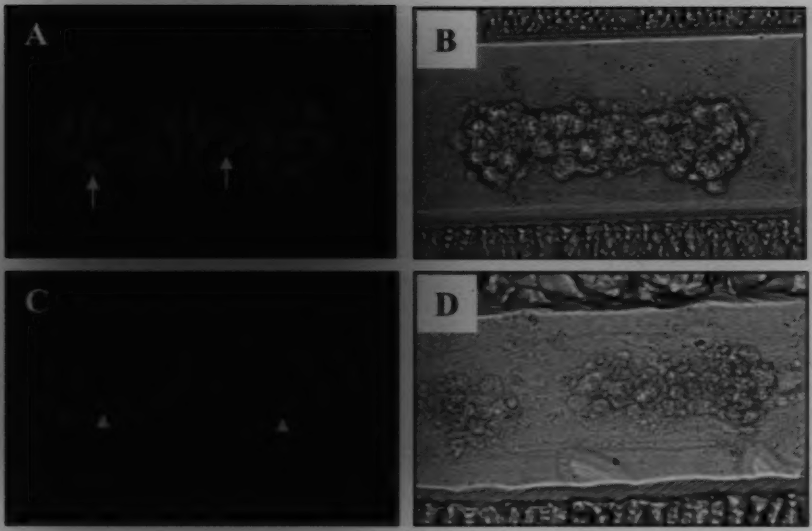


Figure 2-3. Nuclear morphology of non-apoptotic and apoptotic neurons. Compartmented culture neurons were treated for 24 h , then fixed and incubated with Hoescht to stain the nuclei. Representative images of neuronal cell bodies taken with UV filter (A and C) along with matching phase contrast images (B and D). A, B) Cultures in which distal axons were supplied with medium containing 50 ng/ml NGF and cell bodies/proximal axons were supplied with medium lacking NGF, and containing 24 nM NGF antibodies. C, D) Cultures in which the distal axons and the cell bodies/proximal axons were supplied with medium lacking NGF , and containing 24 nM NGF antibodies. Arrows point to non-apoptotic nuclei which are round and cover the majority of the cell body area. Arrowheads point to apoptotic nuclei which are condensed. Condensed nuclei that lie outside of the plane of focus are difficult to visualize and may be absent from images.

2.4 Immunocytochemistry

Compartmented cultures were rinsed with ice-cold Tris-buffered saline (TBS, see Table 2-5 for recipe) at the end of experimental treatments. When probing for phosphorylated proteins, the TBS was supplemented with phosphatase inhibitors, 10 mM sodium fluoride (Sigma) and 2 mM sodium orthovanadate (Sigma), added from stock solutions. The compartments being probed were fixed with 4% (w/v) paraformaldehyde (Fisher) in PBS for 15 min at room temperature. The fixative was removed by rinsing the compartments 3 X 5 min with TBS. Cells were permeabilized with 0.2% Triton X-100 (Sigma) in TBS for 10 min. The Triton-X-100 was removed by 3 X 5 min rinses with TBS supplemented with 0.1% Tween-20 (TBS-T). At this point, the entrance to the proximal compartment was sealed with grease up to the top of the divider. This allowed approximately 100 µl of solution to be retained in the compartment during incubations. For the remaining steps, solutions and rinses were supplied to all compartments, so that any leaks that developed between compartments would not affect the staining procedure. Cultures were blocked with 5.5% normal goat serum (Invitrogen) in TBS-T for 1 h at room temperature or overnight at 4°C. The cultures were then incubated overnight at 4°C with a prepared primary antibody solution: phospho-*c*-jun antibody (Ser63, Cell Signaling) diluted 1:1500 in 3% BSA in TBS, tubulin antibody (Sigma) diluted 1:500 in 3% BSA in TBS, or neurofilament 200 antibody (Sigma) diluted 1:500 in blocking buffer. Following the overnight incubation, cultures were washed 3 X 5 min with TBS-T, then incubated with a biotinylated anti-rabbit antibody diluted 1:500 in TBS , washed 3 X 5 min with TBS-T, then incubated with an avidin+biotinylated horseradish peroxidise (rabbit IgG Vectastain Elite ABC Kit, Vector Labs). Following 3 X 5 min washes with

TBS, immunoreactivity was assayed by the addition of a solution composed of 3,3-diaminobenzidine (DAB) tablets dissolved to a concentration of 0.7 mg/ml in 1.6 mg/ml urea-hydrogen peroxide, 0.06M Tris buffer solution (Sigma). The DAB reacted with the horseradish peroxidise to form a brown precipitate. The peroxidise reaction was stopped by rinsing the cultures with distilled H_2O. After removing the H_2O, the Teflon divider was gently lifted off the dish, and mounting medium followed by a coverslip was overlaid onto the stained cultures. Images were taken with a Nikon D70 or D80 digital camera linked to a Nikon Diapot inverted light microscope. Canon Photostitch software was used to create montages from multiple images.

2.5 Immunoblotting

At the end of the experimental treatments, mass cultures and all compartments of compartmented cultures were rinsed with ice-cold TBS containing 10 mM sodium fluoride and 2 mM sodium orthovanadate. Culture material was harvested with SDS sample buffer (see Table 2-5 for recipe) supplied with phosphatase inhibitors, sodium fluoride (30 mM) and sodium orthovanadate (6 mM). To keep the final extract volumes small, a specialized harvesting technique was employed. For mass cultures, 30 µl of SDS sample buffer was passed from well to well within the same treatment group. A p200 micropipette tip was used to scrape the material off the wells, and the sample buffer was used to wash each well before final collection. Depending on the antigens being probed in an experiment, two or three wells were harvested per group. For compartmented cultures, distal compartments were harvested with the same technique used for mass cultures. The proximal compartments were harvested by supplying 10 µl to each culture in a group, scraping the material with a gel-loading micropipette tip, then pooling the sample buffer from all the cultures into a single sample. For each experiment, 3-6 cultures were harvested per group. When both the CB/PAx and DAx lysates of the same experiment were analyzed, cellular material from an equivalent number of cultures was harvested to obtain the DAx and CB/PAx samples. Immediately after harvesting, 5% (v/v) β-mercaptoethanol (BioShop Canada) was added to the samples that were then boiled for 5 min. If samples needed to be split, the harvesting process was the same except that glycerol was omitted from the 3X SDS sample buffer since it interfered with volume measurements. After the lysates were boiled and separated into two samples, the glycerol was added. Lysates were run on SDS-PAGE mini-gels of the appropriate percentage for resolving the proteins of interest. A lane containing the Page-Ruler Plus pre-stained protein ladder (range 11-250 kDa, Fermentas) was included on every gel for referencing molecular weights (MW). The proteins were wet-transferred in 1 X transfer buffer (see Table 2-5 for recipe) onto Immobilon-P PVDF membranes (Millipore) using a Mini Trans-Blot Electrophoretic transfer cell (Bio-Rad). The membranes were blocked with 5% skim milk powder (Carnation/Safeway) in TBS-T for at least 1 h at room temperature and as long as overnight at 4°C. The primary antibody solutions were prepared in TBS-T containing 5% BSA and 0.05% sodium azide (J.T. Baker) at the dilutions specified in Table 2-3. Primary antibodies were incubated with membranes overnight at 4°C, then the membrane were rinsed several times with TBS-T and further washed a minimum of 3 X 10 min in TBS-T. The appropriate secondary antibody, either goat anti-mouse HRP (Pierce) or goat anti-rabbit HRP (Pierce) was diluted in 5% skim milk powder in TBS-T for a minimum of 45 min at room temperature. The membranes were rinsed and washed by the same procedure used after the primary antibody incubations. Detection was primarily performed using Super Signal West Femto Maximum Sensitivity substrate (Pierce) that can detect as little as a few femtograms of HRP. Some antibodies did not require such a sensitive method of detection and were processed with ECL Western Blotting Detection Reagents (GE Healthcare) instead. BioMax MR film (Kodak) was used to visualize the chemiluminescence signal.

Table 2-3. Specifications of antibodies for immunoblotting

Antibody	**Recognition**[a]	**MW (kDa)**[b]	**Dilution factor**	**2° Ab**[c]	**Clone/ cat #**	**Company**
Actin	β-actin	40	1:1000 -1:2000	M	AC-15 /#A5441	Sigma
Akt, (phospho-)	Akt1 phosphorylated at Ser473, also recognizes corresponding phosphorylation of Akt2 and Akt3	60	1:1000	R	#9271	Cell Signaling
Caspase-3	Full-length p35 and cleaved p17 fragment	35, 17	1:1000	R	#9662	Cell Signaling
c-jun	Total *c*-jun	43, 48	1:1000	R	60A8 /#9165	Cell Signaling
c-jun, (phospho-)	*c*-jun phosphorylated at Ser63	48	1:1000	R	#9261	Cell Signaling
CREB, (phospho-)	CREB phosphorylated at Ser133	43	1:1000	R	#9191	Cell Signaling
Erk	Total Erk1 and Erk2	42, 44	1:2000	R	sc-93	Santa Cruz
Erk, (phospho-)	Human Erk phosphorylated at Thr202 and Tyr204 (Thr183 and Tyr185 for rat Erk)	42, 44	1:1000	R	197G2 /#4377	Cell Signaling
GSK3α, (phospho-)	GSK3α phosphorylated at Ser21	51	1:1000	M	46H12 /#9337	Cell Signaling
GSK3β, (phospho-)	GSK3β phosphorylated at Ser9	46	1:3000	R	#9336	Cell Signaling

Table 2-3. Continued…

Antibody	Recognition[a]	MW (kDa)[b]	Dilution factor	2° Ab[c]	Clone/ cat #	Company
GSK3α/β	Total GSK3 (α and β isoforms)	~47, 51	1:10000 -1:40000	M	1H8/ #368662	Calbiochem
GSK3β	Total GSK3β	46	1:30000	M	#610201	BD Transduction
JNK, (phospho-)	JNK1/2/3 phosphorylated at Thr183 and Tyr185	46, 54	1:40000	R	#9251	Cell Signaling
MKK4, (phospho)	MKK4 phosphorylated at Ser257/Thr261	64	1:10000	R	#9156	Cell Signaling
MKK7, (phospho-)	MKK7 phosphorylated at Ser271/Thr275	48	1:10000	R	#4171	Cell Signaling
PKCδ	Total PKCδ	78	1:20000	R	C-17 /sc213	Santa Cruz
(Ser) PKC substrate, (phospho-)	(R/K)X(S*)(Hyd)(R/K) motif of conventional PKC substrates, *=phosphorylated	N/A	1:10000	R	#2261	Cell Signaling
Trk	Total TrkA, TrkB, TrkC	140	1:10000	R	C14 /sc-11	Santa Cruz
TrkA, (phospho-)	TrkA phosphorylated at Tyr490. Also recognizes TrkB and TrkC phosphorylated at corresponding residues	140	1:10000	R	#9141	Cell Signaling
α-tubulin	α-tubulin	~50	1:20000 -1:50000	M	DM 1A /#T9026	Sigma
β-tubulin	β-tubulin	~50	1:20000 -1:50000	M	TUB 2.1 /#T4026	Sigma

[a] All antibodies reactive to rat, [b] denotes the apparent MW of the protein of interest.

[c] R = rabbit, M = mouse

6

2.6 siRNA transfected cultures

2.6.1 siRNA transfection

Sympathetic neurons were transfected with siRNAs immediately following their dissociation from ganglia. The siRNAs were delivered to the neurons by a technique termed "nucleofection" using a Nucleofector device (Amaxa). Nucleofection is a transfection method that combines both electroporation and proprietary transfection conditions that are optimized for the cell types to be transfected. Multiple siRNAs (Dharmacon) for each rat mRNA target were pooled into a single solution to give a final stock concentration of 200 µM in suspension buffer (Dharmacon). Pools of siRNAs were used to reduce the concentration of each individual siRNA, thereby decreasing the probability of silencing non-specific targets. For GSK3β siRNAs, each siRNA was also tested individually to determine which siRNAs were most effective at silencing GSK3β. The two most effective siRNAs were used in the pool to knockdown GSK3β. Details of each siRNA pool are given in Table 2.4. Assembly of compartmented cultures and collagen coating of well plates were carried out by the standard procedures (Section 2.1.2). On the day of transfection, a modified plating medium consisting of basic medium minus methylcellulose with 50 ng/ml NGF and 2.5% rat serum was pre-incubated in a tissue culture incubator. The collagen-coated well plates were supplied with modified plating medium (500 µl for 24-well plates, 100 µl for 96-well plates) and placed in the incubator. For each transfection group, 20 SCGs were dissociated by the standard procedure described in Section 2.1.3, with the exception that pelleted cells were resuspended in a small amount of dissection medium (0.5 ml for every 20 SCGs processed). The medium was then distributed to sterile RNAse-free microfuge tubes to

0.5 ml aliquots and the cells were gently pelleted at 800 x g. As much of the supernatant as possible was removed without disturbing the pellet, and the cells were gently resuspended in 100 μl of Rat Neuron Nucleofector Solution (Amaxa). A transfection cuvette was prepared by the addition of a small volume of the siRNA stock solution(s). Normally, 10 μl of siRNA stock solution was added to each cuvette to give a final concentration of 18 μM during the transfection. For the (GSK3α + GSK3β) transfections, 10 μl of each siRNA stock solution was added to the cuvette (final transfection concentration = 33 μM). For control transfections with non-targeting siRNA, the final concentration and volume of siRNA matched the experimental groups. For the buffer only control transfection, the volume of RNA suspension buffer was equivalent to the volume used in the experimental groups. The cells suspended in nucleofector solution were added to the transfection cuvette containing the siRNA/buffer, and then the cuvette was placed in the nucleofector device and transfected on setting G-13. Modified plating medium (1.3 ml) was immediately added to the transfection cuvette, and the entire cuvette contents were transferred to a microfuge tube and placed in the incubator for 10 min. The transfected cell suspension was plated in the amounts indicated: 2 ganglia per well for 24-well plates, 0.4 ganglia per well for 96-well plates, 0.35 ganglia per compartmented culture. The following morning, the modified plating medium was replaced with regular culturing medium.

To test the efficiency of uptake of siRNAs using the nucleofection method, sympathetic neurons were transfected with 18 μM of siRNA conjugated to carboxyfluorescein (siGLO Green Transfection Indicator, abs = 494 nm, emission max = 520 nm). The following morning, images were taken on the Leica DMIRE2 microscope with a green filter (excitation filter = 470-540 nm). As shown in Fig. 2-4, all neurons observed by phase contrast were fluorescent, indicating siRNA uptake. In neurons incubated with siGLO Green Transfection Indicator, but not nucleofected, no fluorescent staining was observed in sympathetic neurons.

Table 2-4 siRNAs

siRNA	Target accession number	No. of siRNAs in pool	Catalogue #
Caspase-3	NM_012922	4	D-080028-0,1D-080028-02, D-080028-03, D-080028-04
c-jun	NM_021835	4	L-089158-00
GSK3α	NM_017344	2	J-080107-09 + J-080107-11
GSK3β	NM_032080	4	L-080108-00
Non-targeting	Not applicable	1	D-001810-01
PKCδ	NM_133307	4	D-080142-01, D-080142-02, D-080142-03, D-080142-04
siGLO Green Transfection Indicator	No information	1	D-001630-01

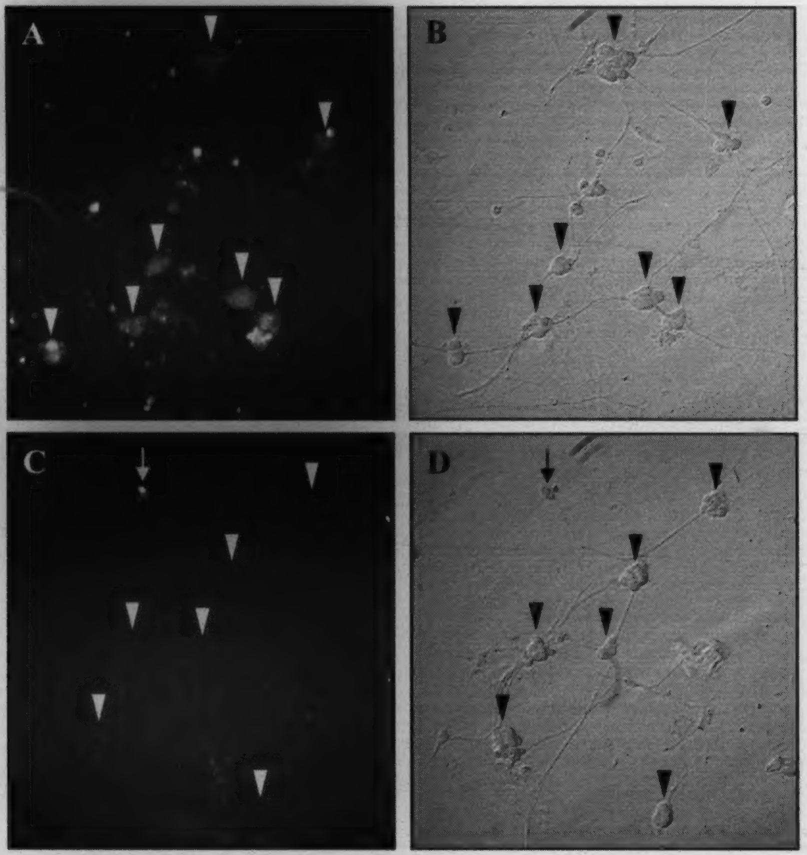


Figure 2-4. Transfection of neurons with fluorescent siRNA. Dissociated sympathetic neurons were mixed with 18 μM of fluorescent siRNA (siGLO Green Transfection Indicator) and transfected by nucleofection (A,B) or not transfected (C,D). The cells were plated in mass culture, incubated overnight, and rinsed prior to imaging. (A, C) Fluorescent images of neurons taken using a green filter (excitation filter = 470-540 nm). (B, D) Matching phase contrast images. Arrowheads indicate clusters of neurons in the image field. Arrows indicate non-neuronal cells that take up the fluorescent siRNA in the absence of nucleofection.

2.6.2 Experimental treatments

Since all experiments for siRNA transfected cultures were carried out between 1 and 6 days of plating, certain modifications were made to the standard protocols. The procedure for treatments in transfected mass cultures is the same described for regular mass cultures (Section 2.2.4), except that the treatment medium was supplemented with 10 μM cytosine arabinoside to kill non-neuronal cells. When measuring neuronal survival by Hoescht stain, the protocol described in Section 2.3 was followed with the exception that the PBS used in the procedure contained 0.2% methylcellulose to minimize detachment of the cells during solution changes.

2.6.3 Immunoblotting

Transfected mass cultures were harvested in JNK lysis buffer supplemented with a Complete Mini protease inhibitor tablet (1 tablet per 10 ml, Roche) to allow for determination of sample protein concentration. The wells were rinsed once on ice in TBS containing 10 mM NaF and 2 mM sodium orthovanadate, after which a small volume of lysis buffer was added to each well. The entire culture plate, on ice, was then placed on an orbital shaker (~150 rpm) for 30 min. The lysis buffer was collected, and the protein concentration determined with the Bio-Rad Protein Assay. Proteins in lysis buffer were prepared for SDS-PAGE by the addition of 5X SDS sample buffer at a dilution factor of 1:5 and 5% (v/v) β-mercaptoethanol. Samples were boiled for 5 min then processed for immunoblotting as described in Section 2.5.

2.7 Axotomy of neurons in compartmented cultures

Neurons in compartmented cultures were axotomized by a combination of mechanical disruption and osmotic lysis. The medium was removed from the distal compartments of two to three cultures at a time. A 5 ml syringe fitted with a 22 gauge needle was filled with sterile double-distilled water. The tip of the needle was positioned inside the distal compartment with the following parameters: at a 45° angle, 3 mm above the dish surface, in front of the axon material, and adjacent and parallel to the barrier separating the distal compartment from the proximal compartment. A short stream of water was ejected from the needle into the compartment. This step was carried out in the other distal compartments. The water in the compartments was aspirated, and two more cycles of water ejection into the compartments were performed. The loss of axonal material in the distal compartment was checked under a light microscope, and injections were repeated as necessary until axon material was no longer visible. The distal compartments were refilled with distal compartment medium and placed in the incubator until the next experimental treatment.

2.8 Assay for retrograde transport of Cy3-labeled NGF

2.8.1 Conjugation of Cy3 to NGF

Lyophilized NGF (250 μg, Alamone) was reconstituted in 250 μl of 0.1M sodium carbonate, pH 9.2. One vial of Cy3 mono-reactive dye (NHS ester reactive group, GE Healthcare) was dissolved in 500 μl of 0.1 M sodium carbonate, pH 9.2, just prior to use. The entire NGF solution was added to 250 μl of the Cy3 mono-reactive dye solution, and the mixture was allowed to incubate for 60 min at room temperature, with mixing every 10 min. A NAP-5 gel filtration column (GE Healthcare) equilibrated with PBS was used to separate the free dye from the Cy3-conjugated NGF. The protein and dye concentration of the purified Cy3-NGF were determined by reading the absorbance at 280 nm and 552 nm, respectively, and converting the measurements with the molar extinction co-efficient of these molecules. The dye:protein ratio was estimated from the calculated concentrations. For the experiments in this thesis the dye:protein ratio was approximately 4:1.

2.8.2 Measuring retrograde transport of Cy3-labeled NGF

Cy3-NGF was added at a final concentration of 50 ng/ml into base medium, which was then supplied to the distal compartments for the treatment times indicated. To determine if Cy3-NGF transport was receptor-mediated, the distal compartments of a control group were provided with 50 ng/ml Cy3-NGF plus a 20-fold-excess of unlabeled NGF (1 μg/ml). Inhibitors were added to the treatment medium prior to incubation as described in Section 2.2.2. Following 6 h of treatment incubation, images of the cell bodies/proximal axons were taken on a Leica DMIRE2 microscope with a red filter (excitation filter = 560-640 nm).

Table 2-5 Standard Buffers and solutions

Name	Composition
1 X gel running buffer	25 mM Tris, 190 mM glycine, 0.1% SDS
1 X transfer buffer	25 mM Tris, 190 mM glycine, 20% (v/v) methanol
3X SDS sample buffer	6% SDS, 240 mM Tris-HCl, pH 6.8, 30% (v/v) glycerol, bromophenol blue to colour
JNK lysis buffer	25 mM HEPES, pH 7.5, 20 mM β-glycerophosphate, 0.3 M NaCl, 1.5 mM $MgCl_2$, 0.1% Triton X-100, 0.5 mM DTT, 20 mM NaF, 2 mM $NaVO_4$
Mounting medium	70% (v/v) glycerol, 20 mM Tris, 135 mM NaCl, pH 7.5
PBS	135 mM NaCl, 2.5 mM KCl, 1.5 mM KH_2PO_4, 10 mM $NaH_2PO_4 \cdot H_2O$, pH 7.2
SDS-PAGE gel	8-12% bis-acrylamide (1:29), 375 mM Tris-HCl, pH 8.8, 0.1% SDS, 0.04% APS, 0.1% TEMED
sodium fluoride stock	0.95 M NaF

Table 2-5. Continued…

Name	Composition
sodium orthovanadate stock	200 mM $NaVO_4$, pH 10. Boil until colorless.
TBS	20 mM Tris, 135 mM NaCl, pH 7.5
TBS-T	20 mM Tris, 135 mM NaCl, pH 7.5, 0.1% Tween-20

Table 2-6. Sources for materials, chemicals, and reagents

Item	Source
Media and culturing materials	
2.5S Nerve Growth Factor	Alamone
6,7-dimethyl-5,6,7,8-tetrahydropterine	Sigma
choline chloride	Sigma
Coenzyme A	Sigma
collagenase type 1 from *Costridium histolyticum*	Sigma
cytosine 3-*D*-arabino-furanoside	Sigma
D-Biotin	Calbiochem
D-glucose	Sigma
fumaric acid	Sigma
glutathione	Sigma
imidazole	Sigma
L-15 medium powder w/ glutamine, w/o sodium bicarbonate	Invitrogen
L-ascorbic acid	Sigma
L-aspartic acid	Sigma
L-cystine	Sigma
L-glutamic acid	Sigma
L-glutamine, 200 mM	Sigma
lipoic acid	Sigma
L-proline	Sigma
methylcellulose, 4000 c.p.s.	Xenex Laboratories
myo-inositol	Sigma
$NaHCO_3$	Sigma
P-aminobenzoic acid	Sigma
Penicillin-Streptomycin solution, 10 000 U ml^{-1}	Sigma
phenol red	Sigma
rat serum	prepared in laboratory (Campenot et al. 2009)

Table 2-6. Continued…

Item	Source
rat tail collagen	Sigma, or prepared in laboratory (Campenot et al. 2009)
silicone grease, high-vacuum	Dow Corning
Teflon divider	Tyler Research
trypsin	Sigma
Vitamin B12	Sigma
β-alanine	Sigma
Experimental treatments	
anti-NGF	Cedarlane
Chelerythrine	Calbiochem
Colchicine	Sigma
Cy3 mono-reactive dye	GE Healthcare
DMSO (dimethyl sulfoxide)	Sigma
Gö6976	Calbiochem
K252a	Calbiochem
Kenpaullone	Calbiochem
LiCl	Sigma
NaCl	EMD
NAP-5 gel filtration column	GE Healthcare
NSC23766	Calbiochem
rottlerin	Calbiochem
Post-treatment	
40% bis-acrylamide	Bio-Rad
BSA	Sigma
DAB	Sigma
ECL Western Blotting Detection Reagents	GE Healthcare
Glycerol	Anachemia
Glycine	Sigma/Fisher
Goat serum	Invitrogen
Hoechst 33258	Molecular Probes
Immobilon-P PVDF	Millipore
KCl	Sigma
KH_2PO_4	Fisher
BioMax MR film	Kodak
Methanol	Fisher

Table 2-6. Continued…

Item	Source
NaCl	EMD
$NaH_2PO_4 \bullet H_2O$	Sigma
Paraformaldehyde	Fisher
PBS	Invitrogen
Pre-stained molecular weight markers (11-250 kDa)	Fermentas
rabbit IgG Vectastain Elite ABC Kit	Vector Labs
SDS	Bio-Rad
Skim milk powder	Carnation/Safeway
Sodium azide	J.T. Baker
Sodium fluoride (NaF)	Sigma
Sodium orthovanadate ($NaVO_4$)	Sigma
Super Signal West Femto Maximum Sensitivity substrate	Pierce
Tris, Ultra Pure	Invitrogen
Tris-HCl	EMD
Triton X-100	Sigma
Tween-20	Fisher
β-mercaptoethanol	BioShop Canada
siRNA transfections and treatments	
Complete Mini protease inhibitor tablet	Roche
DTT	Fisher
HEPES	Fisher
$MgCl_2$	EMD
Rat Neuron Nucleofector kit	Amaxa
β-glycerophosphate	Sigma

CHAPTER 3:

AN NGF RETROGRADE SURVIVAL SIGNAL THAT IS MEDIATED BY MECHANISMS DOWNSTREAM OF TRKA

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3.1 Rationale

An NGF retrograde survival signal that has been predicted by the signaling endosome hypothesis is composed of endosomes carrying NGF and phosphorylated TrkA. Upon arriving at the cell bodies, this retrograde signal is proposed to utilize the phosphorylated TrkA to mediate downstream signaling that promotes survival. In a study carried out in compartmented-cultured sympathetic neurons treated with the Trk inhibitor, K252a, it was found that TrkA phosphorylation in the cell bodies/proximal axons was critical for survival mediated by NGF retrograde signals (Ye et al., 2003). The collective results of this study were interpreted as evidence that an NGF retrograde signal consisting of endosomes carrying NGF and TrkA serves a vital role in promoting NGF-dependent survival. In contrast, MacInnis et al. (2003) carried out similar experiments with compartmented-cultured sympathetic neurons and the Trk inhibitor, K252a, and observed the NGF retrograde signaling could fully support survival in the absence of TrkA phosphorylation in the cell bodies/proximal axons. The results of this study conflicted with the conclusions of Ye et al. (2003) since they indicated that NGF was fully capable of promoting survival via retrograde survival signals that did not require phosphorylated TrkA in the cell bodies/proximal axons for their actions. To add to the controversy created by the conflicting reports it was also found that the Trk inhibitor used in these studies, K252a, had a survival-promoting side effect at higher concentrations that complicated data interpretation (MacInnis et al., 2003).

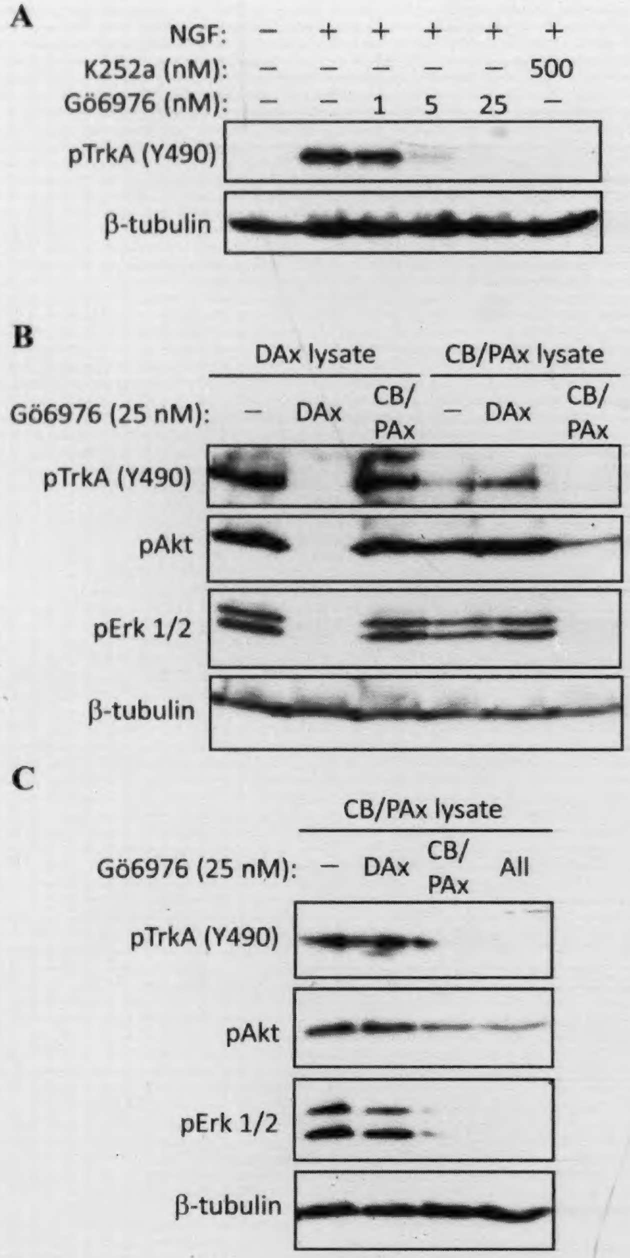
The identification of another Trk inhibitor, Gö6976 (Behrens et al., 1999), provided a new opportunity for determining whether or not phosphorylated TrkA in the cell bodies is required for NGF retrograde survival signaling. It was found that NGF retrograde signaling fully supported survival when TrkA phosphorylation in the cell bodies/proximal axons was inhibited using Gö6976. Consistent with this result, inhibition of TrkA phosphorylation in the cell bodies/proximal axons also did not block NGF retrograde signaling from activating the downstream pro-survival signaling molecules, Akt and CREB, in the cell bodies/proximal axons. Providing justification for the use of Gö6976 in the present study and for future Trk inhibitor studies, it was demonstrated that at concentrations of Gö6976 which effectively inhibit TrkA phosphorylation, Gö6976 does not share K252a's survival-promoting side effect.

3.2 Results

3.2.1 Gö6976 inhibits NGF-induced phosphorylation of TrkA in sympathetic neuron cultures

It was previously determined that NGF-induced tyrosine phosphorylation of TrkA was inhibited with as little as 1 nM Gö6976 in the GT1-1-trk9 cell line (Behrens et al., 1999). However, for the present study, the concentration of Gö6976 required to inhibit TrkA phosphorylation in sympathetic neurons needed to be established. Mass cultures were pre-incubated in base medium lacking NGF for 30 min to aid in the removal of residual NGF from the culturing medium. The mass cultures were then treated with medium containing no NGF or 50 ng/ml NGF in the presence of 1, 5, or 25 nM Gö6976 or 500 nM K252a for 8 h as indicated in Figure 3-1A. The treated cultures were harvested and analyzed by immunoblot for the phosphorylation of TrkA at tyrosine 490 (Y490). The phosphorylation state of Y490 was chosen for analysis because it regulates the binding of adaptor proteins implicated in promoting activation of downstream survival signaling such as the PI3K/Akt pathway (Atwal et al., 2000 and Section 1.4.2). For

Figure 3-1. Gö6976 inhibits phosphorylation of TrkA in cultures of rat sympathetic neurons. A) Dose-dependent inhibition of TrkA phosphorylation by Gö6976 in mass cultures. Mass cultures were incubated for half an hour in medium without NGF before treatment for 8 h with medium containing no NGF or 50 ng/ml NGF plus 1, 5, or 25 nM Gö6976 or 500 nM K252a. Lysates were analyzed by immunoblot for TrkA phosphorylated at Tyr490 (pTrkA, Y490) and the loading control, β-tubulin. Results are representative of 3 experiments. B and C) Compartmented cultures were supplied with 200 ng/ml NGF in all compartments for 1 h. Gö6976 (25 nM) was concurrently supplied to the cell bodies/proximal axons (CB/PAx), the distal axons (DAX), or all compartments (All) as indicated for the duration of the treatment. The lysates were analyzed by immunoblot for phosphorylation of TrkA (pTrkA, Y490), Akt (pAkt, S473), and Erk1/2 (pErk, T202/Y204). The blots were stripped and reprobed for β-tubulin as a loading control. Results are representative of 3 experiments. B) Gö6976 inhibition of TrkA is confined to the compartment the inhibitor is supplied to. C) Residual phosphorylation of Akt in the cell bodies/proximal axons is observed in cultures treated with Gö6976 in either the CB/PAx or all compartments.


A
NGF: − + + + + +
K252a (nM): − − − − − 500
Gö6976 (nM): − − 1 5 25 −
pTrkA (Y490)
β-tubulin
B
DAx lysate
CB/PAx lysate
Gö6976 (25 nM): − DAx CB/PAx − DAx CB/PAx
pTrkA (Y490)
pAkt
pErk 1/2
β-tubulin
C
CB/PAx lysate
Gö6976 (25 nM): − DAx CB/PAx All
pTrkA (Y490)
pAkt
pErk 1/2
β-tubulin

control cultures treated with 50 ng/ml NGF, an intense signal for TrkA phosphorylated at Y490 compared to cultures incubated in medium without NGF was observed (Figure 3-1A). A dose-dependent loss of TrkA phosphorylation (Y490) was observed when cultures in 50 ng/ml NGF were co-treated with increasing concentrations of Gö6976 (1, 5, 25 nM). At 25 nM Gö6976, phosphorylation of TrkA at Y490 was undetectable, similar to the no NGF control group. NGF induction of TrkA phosphorylation at Y490 was also potently inhibited by 500 nM K252a, which was in line with previous experiments showing that 500 nM K252a inhibited phosphorylation of two other TrkA phosphorylation sites, Y674 and Y675 (MacInnis et al., 2003).

Before proceeding with the study it was important to verify that Gö6976 could be used to inhibit TrkA phosphorylation in the individual compartments of compartmented cultures i.e. if Gö6976 inhibited TrkA phosphorylation only in the compartment in which it was applied. To set up this experiment, activation of TrkA was induced in both the cell bodies/proximal axons (CB/PAx) and the distal axons (DAx) by supplying 200ng/ml NGF to all compartments of the compartmented cultures. Gö6976 (25 nM) was concurrently supplied to either the CB/PAx or DAx, and the cultures were incubated for 1 h prior to being harvested and analyzed by immunoblot. In control cultures stimulated with 200 ng/ml NGF but without Gö6976, TrkA was phosphorylated at Y490 in both the distal axon and cell bodies/proximal axon lysates (Figure 3-1B). Treatment of the distal axons with Gö6976 resulted in a dramatic loss in TrkA phosphorylation in the distal axons without decreasing TrkA phosphorylation of the cell bodies/proximal axons. Conversely, application of Gö6976 to the cell bodies/proximal axons compartments only decreased the phosphorylation of TrkA in the cell bodies/proximal axons and not in the distal axons. These results indicated that Gö6976's inhibitory effect on TrkA phosphorylation was restricted to the compartment to which Gö6976 was supplied. In this experiment, the phosphorylation of two molecules downstream of TrkA activation, Akt and Erk1/2, were also examined (Andjelkovic et al., 1998; Creedon et al., 1996). Akt phosphorylation at Ser473 is required for its full activation (Alessi et al., 1996). Erk1 and Erk2 are activated by analogous threonine and tyrosine phosphorylation: Thr202 and Tyr204 for Erk1, Thr185 and Tyr 187 for Erk2 (Anderson et al., 1990; Boulton et al., 1991; Payne et al., 1991). Phosphorylation of both Akt and Erk1/2 was observed in both the distal axons and cell bodies/proximal axons stimulated by application of 200 ng/ml NGF. When TrkA phosphorylation in the distal axons was inhibited with 25 nM Gö6976, it resulted in the concurrent loss of phosphorylation of both Akt and Erk1/2 in the distal axons, exclusively. The phosphorylation of these molecules in the corresponding cell bodies/proximal axons was not decreased. Consistent with this result, inhibition of TrkA phosphorylation in the cell bodies/proximal axons with Gö6976 resulted in a local decrease in Akt and Erk phosphorylation without affecting the phosphorylation of these molecules in the distal axons. Interestingly, there was a residual signal for phosphorylated Akt in cell bodies/proximal axons treated directly with Gö6976. This residual phosphorylation was not detected in the distal axons when they were directly treated with Gö6976. One possible explanation for these results is that in cultures treated with Gö6976 on the cell bodies/proximal axons, NGF is still capable of activating TrkA in the distal axons and generating downstream signals, some of which may be retrogradely transmitted to the cell bodies to activate Akt. It is also plausible that Akt activated in the distal axons may itself be retrogradely transmitted to the cell bodies. However, when cultures were treated with Gö6976 in all compartments for 1 h to block all sources of TrkA activity, the residual phosphorylation of Akt in the cell bodies/proximal axons persisted (Figure 3-1C), arguing against its activation by a retrograde signal initiated by TrkA in the distal axons. Instead, the data would suggest that at the time point examined (1 h), the Akt in the cell bodies/proximal axons may be partially resistant to dephosphorylation compared to the Akt in distal axons. However, it is important to note that these findings do not rule out a mechanism whereby TrkA in distal axons does induce retrograde activation of Akt in the cell bodies/proximal axons, since there is evidence for this mechanism when longer time points are examined later in this study (Figure 3-4).

3.2.2 Inhibiting TrkA phosphorylation in the cell bodies and proximal axons does not prevent NGF signaling at the distal axons from supporting survival

The finding that Gö6976 could be used to locally inhibit TrkA phosphorylation in the cell bodies/proximal axons or distal axons of compartmented cultures provided an excellent opportunity to test if TrkA phosphorylation in either the distal axons or the cell bodies/proximal axons is necessary for the survival mediated by NGF retrograde signaling. To produce experimental conditions under which sympathetic neuron survival was dependent on NGF retrograde signaling, NGF (50 ng/ml) was only supplied to the distal axons of sympathetic neurons in compartmented culture. The cell bodies/proximal axons of the cultures were supplied with medium containing NGF antibodies to block any residual NGF from signaling within the compartment. For the duration of the experimental treatments, 1, 5, or 25 nM Gö6976 was applied to the distal axons or alternatively, 25 nM of Gö6976 was applied to the cell bodies/proximal axons. One culture group was not supplied with any NGF to determine the baseline survival rate in the absence of NGF retrograde signals. The cultures were treated for 48 h before being fixed and Hoescht stained. Neuronal survival rates were determined by analyzing the morphology of nuclei (see Section 2.3). A round full nucleus was indicative of a surviving neuron, whereas a condensed nucleus was indicative of a neuron that had undergone apoptosis during the experiment. In control cultures supplied with 50 ng/ml NGF on the distal axons, 97% of the neurons survived (Figure 3-2A). In contrast, only 22% of neurons survived in cultures completely deprived of NGF. In cultures treated with Gö6976 at the distal axons, 1 nM Gö6976 did not significantly decrease survival (96%) compared to the NGF control group. In contrast, 5 nM Gö6976 reduced survival to 55%, and 25 nM Gö6976 reduced the survival rate to only 20%, a rate comparable to that observed in NGF-deprived cultures (22%). The ability of a concentration of Gö6976 to reduce survival correlated with its ability to inhibit TrkA phosphorylation (Figure 3-1A). These results were expected since NGF mediates its survival promoting actions through binding and activation of TrkA, and application of Gö6976 to the distal axons, where the NGF is localized, would prevent the initiation of all TrkA signaling and therefore retrograde signaling in the these cultures.

It was next assessed if TrkA phosphorylation in the cell bodies/proximal axons was required for the survival mediated by NGF retrograde signaling. A concentration of 25 nM Gö6976 was tested because it was the concentration that blocked TrkA phosphorylation most effectively. When neurons were supplied with NGF only on their distal axons, application of 25 nM Gö6976 to the cell bodies/proximal axons resulted in the survival of 91% of neurons (Figure 3-2A), which was not significantly different from

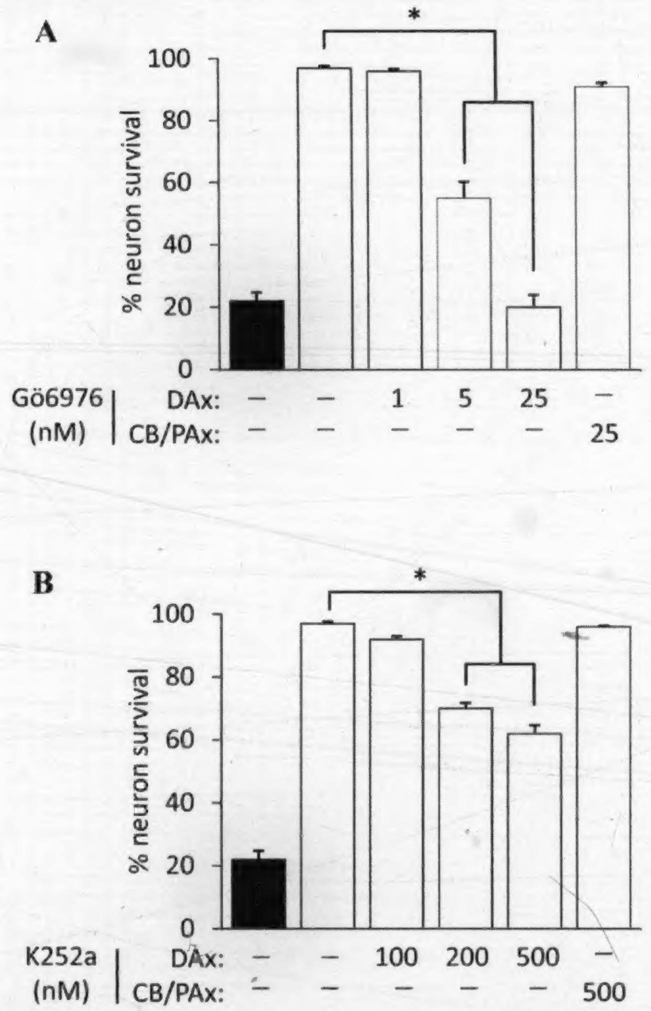


Figure 3-2. Comparing the effects of TrkA inhibition by Gö6976 and K252a on survival mediated by NGF retrograde signals. Compartmented cultures were treated with medium containing anti-NGF at the cell bodies/proximal axons (CB/Pax) and 50 ng/ml NGF (white bars) or anti-NGF (black bars) at the distal axons (DAx). Concurrently, the cultures were supplied with Gö6976 or K252a at their DAx or CB/PAx at the concentrations indicated. Survival was assessed after 48 h of treatment by examining Hoescht-stained nuclei. The mean percent survival from data compiled from 3 experiments is graphed (n = 8, ± SEM). * = ($p < 0.001$) in an unpaired sample t-test. A) Quantification of neuronal survival in cultures treated with Gö6976. B) Quantification of neuronal survival in cultures treated with K252a.

the rate obtained for the NGF control group (97%). The previous observation that 25 nM Gö6976 potently inhibited survival when supplied to the distal axons receiving the NGF suggests that this concentration of Gö6976 is effective at inhibiting NGF survival signaling mediated by TrkA. Therefore, the lack of any significant reduction in survival when 25 nM Gö6976 was supplied to the cell bodies/proximal axons of neurons supplied with NGF on their distal axons is a strong indication that NGF initiates retrograde signaling that does not require the phosphorylation of TrkA in the proximal axons or the cell bodies in order to mediate survival. These results support the findings of MacInnis et al. (2003) but not Ye et al. (2003) which examined the requirements of TrkA phosphorylation using the Trk inhibitor, K252a.

3.2.3 Gö6976 does not promote survival, a side effect observed with the Trk inhibitor, K252a

Since K252a is the most commonly used Trk inhibitor in studies to date, carrying out experiments in which Gö6976 and K252a can be directly compared may reveal if there are differences between the actions of the inhibitors. These differences may indicate that one inhibitor is more suitable for use in future studies. In the present study, K252a was tested in parallel with Gö6976 in the survival experiment described in the previous Section. The concentrations of K252a tested were the same as those used in MacInnis et al. (2003). The results with K252a are shown in Figure 3-2B. When 50 ng/ml of NGF was supplied to distal axons, the concurrent application of 100 nM K252a to the distal axons resulted in a survival rate of 92% which was not significantly lower than the NGF control group (97%). When 200 nM or 500 nM of K252a were applied to the distal axons, the survival rate of the neurons dropped significantly to 70% and 62%, respectively. Application of 500 nM K252a to the cell bodies/proximal axons did not significantly decrease neuron survival (96%). The results of the experiment were similar to those obtained by MacInnis et al. (2003) with the exception that in the previous report, application of 100 nM K252a to the distal axons did significantly reduce survival. One reason for this discrepancy may be that in the present study, 50 ng/ml of NGF was supplied to the distal axons, whereas in MacInnis et al. (2003) only 10 ng/ml of NGF was supplied. The level of inhibition of TrkA achieved with 100 nM K252a may have been sufficient to block a significant portion of the NGF signaling produced by lower concentrations of NGF (10 ng/ml) but not higher concentrations (50 ng/ml).

The results obtained with K252a treatment revealed that there was still a large percentage of neurons that survived when 500 nM K252a was applied to the distal axons (62%) compared to the control group completely deprived of NGF (22%) even though immunoblotting showed that 500 nM of K252a reduced TrkA phosphorylation to the level observed for neurons not supplied with NGF or for neurons treated with 25 nM Gö6976 (Figure 3-1A). In Figure 3-2A, it was demonstrated that application of 25 nM Gö6976 to the distal axons decreased survival to 20%, a rate that did not differ significantly from NGF-deprived control cultures (22%). An explanation offered in MacInnis et al. (2003) to explain the discrepancy between K252a's ability to inhibit TrkA phosphorylation and its effects on survival was that, at high concentrations, K252a produced a survival promoting side-effect that was independent and counteractive to the effects caused by the inhibition of TrkA phosphorylation. This idea stemmed from several previous studies which reported that K252a had neuroprotective effects (Borasio, 1990; Cheng et al., 1994; Glicksman et al., 1995). Indeed, when MacInnis et al. (2003)

completely deprived compartmented cultures of NGF, application of 500 nM K252a to the cell bodies/proximal axons still significantly increased the rate of survival compared to no drug controls, suggesting that K252a affected signaling events in a manner that promoted survival. The survival outcome could be achieved if K252a induced survival signaling or, alternatively, if K252a prevented apoptotic signaling.

The ability of K252a to promote survival understandably poses potential problems for investigations using K252a to study the effects of Trk inhibition on survival. Therefore, it was important to determine whether or not Gö6976 had a similar survival promoting ability. To test this, compartmented cultures were deprived of all NGF support in the absence or presence of K252a and/or Gö6976 at the cell bodies/proximal axons. Survival at 48 h was assessed by examining nuclear morphology. As expected from the previous report of MacInnis et al. (2003), application of 500 nM K252a to the cell bodies/proximal axons significantly increased the survival rate to 53% compared to the only 22% survival detected in the no drug control group (Figure 3-3A). Importantly, no increase in survival was observed when 25 nM Gö6976 was supplied to the cell bodies/proximal axons (17% survival) compared to the no drug controls, which indicates that, at the concentration tested, Gö6976 does not produce the survival promoting side-effect observed with 500 nM K252a. This is an important finding because it indicates that, in the experiment where NGF is applied to the distal axons and Gö6976 is applied to the cell bodies/proximal axons (Figure 3-2A), the strong rate of neuronal survival observed (91%) is due to the actions of NGF retrograde signals rather than survival signals induced by Gö6976 at the cell bodies/proximal axons. The results continue to support the concept that NGF produces retrograde signaling that can effectively promote

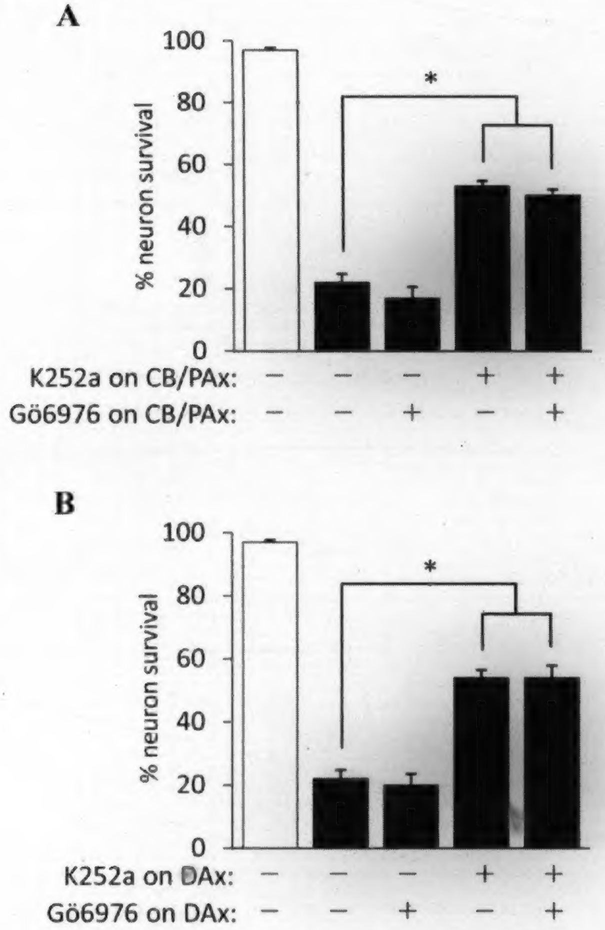


Figure 3-3. Unlike K252a, Gö6976 does not promote survival in the absence of NGF signaling. Compartmented cultures were treated with medium containing anti-NGF at the cell bodies/proximal axons (CB/Pax) and 50 ng/ml NGF (white bars) or anti-NGF (black bars) at the distal axons (DAx). Concurrently, the cultures were supplied with 25 nM Gö6976 and/or 500 nM K252a at their DAx or CB/PAx, as indicated. Survival was assessed after 48 h of treatment by examining Hoescht-stained nuclei. The mean percent survival from data compiled from 3 experiments is graphed (n = 8, ± SEM). * = ($p <$ 0.001) in an unpaired sample t-test. A) Quantification of neuronal survival in NGF-deprived cultures treated with Gö6976 and/or K252a on their cell bodies/proximal axons. B) Quantification of neuronal survival in NGF-deprived cultures treated with Gö6976 and/or K252a on their distal axons.

survival and which does not require TrkA phosphorylation in the cell bodies and proximal axons to do so.

The survival of NGF-deprived cultures in which 500 nM K252a and/or 25 nM Gö6976 was applied to the distal axons was also examined (Figure 3-3B). Similar to the results described above, only the presence of 500 nM K252a increased the survival (54%) of neurons compared to the no NGF controls (22%). This was an intriguing result since it indicated that K252a could support survival by altering signaling in the distal axons, a situation that resembles the ability of NGF to support survival when applied only to the distal axons. It is unlikely that K252a somehow mediates it effects by acting on TrkA, since co-application of the alternate Trk inhibitor, Gö6976, did not significantly lower the survival rate compared to cultures treated with K252a alone. Based on these data, it is possible that K252a produces its survival effects by acting on signaling events downstream of TrkA in the distal axons in a manner that mimics NGF-dependent retrograde signaling.

3.2.4 Inhibition of TrkA at the cell bodies/proximal axons with Gö6976 does not prevent the phosphorylation of the TrkA downstream effectors, Akt and CREB

The results of the present study have thus far indicated that NGF signaling at the distal axons is sufficient to support survival and that the signaling does not require the phosphorylation of TrkA at the cell bodies or proximal axons to operate. One hypothesis for how NGF signaling at the distal axons may promote survival is through the retrograde transmission of survival signals to the cell bodies. A retrograde survival signal that is predicted by the "signaling endosome hypothesis" is an endosome containing NGF and phosphorylated TrkA. However, this signal would require TrkA phosphorylation in the cell bodies/proximal axons to promote downstream survival signaling, and the Gö6976 experiments of this study reveal that survival can be fully supported without TrkA phosphorylation in the cell bodies/proximal axons. Therefore, it is predicted that other NGF retrograde survival signals are operating within sympathetic neurons to promote survival. It is known that TrkA activated in the distal axons induces downstream signaling events locally (Kuruvilla et al., 2000; MacInnis et al., 2003, and Figure 3-2B) Thus, one possibility is that some of these downstream signals are transmitted to the cell bodies where they mediate further downstream survival signaling without phosphorylated TrkA. If this hypothesis is valid, there should be evidence of activation of survival signaling events, downstream of TrkA, in the cell bodies/proximal axons.

To test the above hypothesis, the activation of Akt and CREB was examined since these molecules are implicated in mediating NGF-dependent survival in sympathetic neurons (Crowder and Freeman, 1998; Riccio et al., 1999). Moreover, since CREB is a transcription factor, it is likely that its activation will occur in the cell body where the nucleus is located. Phosphorylation of Akt on Ser473 (Alessi et al., 1996) and CREB on Ser133 (Gonzalez and Montminy, 1989) is associated with their activation. Compartmented cultures were either treated with 50 ng/ml of NGF only on their distal axons or deprived of NGF in all compartments. During the experiment, some treatment groups were also supplied with 25 nM Gö6976 on the cell bodies/proximal axons. After 24 h of treatment, lysates from all compartments were analyzed by immunoblot. Cultures supplied with NGF on distal axons produced strong signals for both phosphorylated TrkA and phosphorylated Akt in the distal axons compared to cultures completely deprived of NGF (Figure 3-4), consistent with previous reports (Kuruvilla et al., 2000; MacInnis

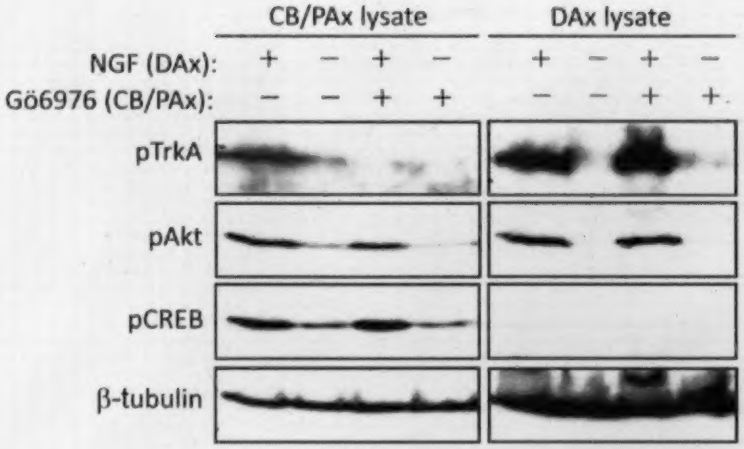


Figure 3-4. Inhibition of TrkA phosphorylation in the cell bodies/proximal axons with Gö6976 does not prevent NGF-induced retrograde phosphorylation of Akt and CREB. Compartmented cultures were treated with medium containing anti-NGF at the cell bodies/proximal axons (CB/Pax) and 50 ng/ml NGF (+) or anti-NGF (-) at the distal axons (DAx). Concurrently, the CB/PAx were supplied with 25 nM Gö6976 where indicated. Lysates from cultures treated for 24 h were analyzed by immunoblot for phosphorylation of TrkA (pTrkA, Y490), Akt (pAkt, S473), and CREB (pCREB, S133). The blot was stripped and reprobed for β-tubulin as a loading control. Results are representative of 3 experiments.

et al., 2003). Phosphorylated CREB was not detectable in the distal axons under any condition examined. Supplying cultures with NGF on the distal axons also resulted in increased phosphorylation of TrkA, Akt, and CREB in the cell bodies/proximal axons compared to cultures completely deprived of NGF suggesting that these molecules were activated as a result of retrograde signaling initiated by NGF in the distal axons. Mechanisms previously proposed to account for the phosphorylation of TrkA in the cell bodies/proximal axons include the internalization and retrograde transport of phosphorylated TrkA from the distal axons (Grimes et al., 1996; Hendry et al., 1974; Howe and Mobley, 2004) or the lateral propagation of TrkA activation in the plasma membrane (Senger and Campenot, 1997). To determine if the phosphorylation of TrkA in the cell bodies/proximal axons was responsible for the observed increase in phosphorylation of Akt and CREB, Gö6976 was applied to the cell bodies/proximal axons. In cultures supplied with NGF on distal axons but treated with Gö6976 on the cell bodies/proximal axons, TrkA phosphorylation was locally inhibited in the cell bodies/proximal axons as expected. However, the levels of Akt and CREB phosphorylation throughout the neuron were unaffected; Gö6976 on the cell bodies/proximal axons did not prevent the phosphorylation of TrkA and Akt in the distal axons and notably, did not block the phosphorylation of Akt and CREB in the cell bodies/proximal axons. Application of Gö6976 to the cell bodies/proximal axons in cultures completely deprived of NGF did not result in phosphorylation of TrkA, Akt or CREB in any compartment indicating that the Gö6976 drug itself did not cause the activation of these molecules. These results demonstrate that, despite the loss of phosphorylated TrkA in the cell bodies and proximal axons, Akt and CREB are still phosphorylated in the cell bodies/proximal axons via signaling initiated by NGF at the distal axons. These data are consistent with the hypothesis that signaling molecules activated downstream of TrkA in the distal axons function as retrograde survival signals that are transmitted to the cell bodies to mediate local activation of signaling events implicated in promoting neuron survival.

3.3 Discussion

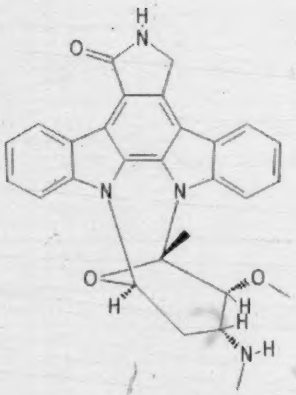
In this study, the requirements of TrkA phosphorylation in mediating NGF retrograde survival signaling in sympathetic neurons was investigated using the Trk inhibitor, Gö6976. The results provide evidence that the survival of sympathetic neurons is completely supported by NGF retrograde signaling that does not require the phosphorylation of TrkA in the proximal axons or the cell bodies to carry out its survival-promoting actions. The ability of NGF signaling from the distal axons to induce phosphorylation of Akt and CREB in the cell bodies/proximal axons, even when TrkA phosphorylation in the cell bodies/proximal axons is blocked, indicates that the retrograde survival signals responsible for generating these downstream signaling events do not utilize phosphorylated TrkA in the proximal axons leading to the cell bodies, or in the cell bodies themselves, to effectively produce their effects. These retrograde survival signals may instead be composed of molecules activated downstream of TrkA in the distal axons that, when transmitted to the cell bodies, are capable of mediating signaling that promotes survival.

3.3.1 Comparing the actions of K252a and Gö6976

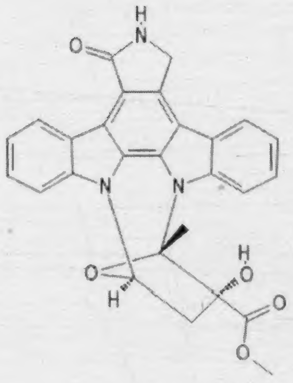
Previous studies have commonly employed K252a to inhibit TrkA phosphorylation. However, it has been reported that treatment of neurons with K252a also induces pro-survival effects (Borasio, 1990; Cheng et al., 1994; Glicksman et al., 1995). Consistent with these reports, it was demonstrated that in sympathetic neurons, high concentrations of K252a (500 nM) promoted a significant amount of neuron survival, even when the neurons were not supplied with any NGF (MacInnis et al., 2003). This pro-survival side effect of K252a complicates the interpretation of experiments designed to examine the effects of TrkA inhibition on survival.

It was important to determine that the Trk inhibitor employed in this study, Gö6976, did not possess a survival promoting effect similar to that observed with K252a. Experiments carried out with K252a reconfirmed that 500 nM K252a mediated neuron survival in cultures completely deprived of NGF. This survival effect was observed when K252a was applied to either the distal axons or the cell bodies/proximal axons. In contrast, when a concentration of Gö6976 (25 nM) that effectively inhibited TrkA phosphorylation was applied to either the distal axons or cell bodies/proximal axons of NGF-deprived cultures, no significant increase in survival was observed compared to cultures deprived of NGF in the absence of Gö6976. These results indicate that Gö6976 does not share K252a's survival-promoting side effect. In light of these findings, Gö6976 should be considered for future studies examining the effects of Trk inhibition on survival.

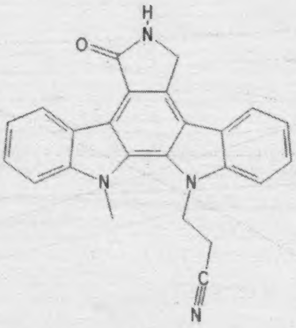
The result that Gö6976 does not share K252a's survival-promoting effect may be surprising considering that the two inhibitors are so closely related in their structure (Figure 3-5). Both K252a and Gö6976 are also structurally related to another well known inhibitor compound, staurosporine. All three inhibitors have been classified as belonging to a family of compounds called indolocarbazoles (reviewed by Sanchez et al., 2006).



Staurosporine



K252a



Gö6976

Figure 3-5. Chemical structures for staurosporine, K252a, and Gö6976. Structures were downloaded from the Pubchem database: http://www.ncbi.nlm.nih.gov/sites/entrez?db=pcsubstance

The three compounds were all originally recognized for their ability to inhibit members of the protein kinase C (PKC) family (Kase et al., 1986; Martiny-Baron et al., 1993; Tamaoki et al., 1986) but were subsequently found to be potent inhibitors of Trk receptors as well (Behrens et al., 1999; Ohmichi et al., 1992; Tapley et al., 1992). A common mechanism of inhibition of protein kinases by indolocarbazoles is via occupation of the ATP-binding site (reviewed by Sanchez et al., 2006). Consistent with this mechanism, inhibition of TrkA by K252a is competitive with respect to ATP (Angeles et al., 1998) and it is probable that Gö6976 inhibits TrkA by this same mechanism as well. Why then are differential effects observed with K252a and Gö6976 in the experiments performed in this study? It is likely that the small differences in the structures of these two compounds affect their ability to inhibit targets other than TrkA. Indeed, studies with K252a have shown that small modifications of the compound can be made that enhance specificity for some targets and decrease specificity for other targets (Hudkins et al., 2007). Therefore, although Gö6976 and K252a share an ability to inhibit TrkA, Gö6976 may lack the ability to affect an alternate target through which K252a produces its pro-survival effect. It is highly unlikely that K252a mediates its survival promoting activity by acting on TrkA, due to the data presented in this study that co-application of Gö6976 and K252a to neurons deprived of NGF did not result in a decreased survival rate compared to neurons treated with K252a alone. Since under these conditions it is expected that Gö6976 and K252 would compete for the same binding site on TrkA, if K252a did promote survival by acting on TrkA there should have been at least some loss of survival observed in the presence of Gö6976 due to the occupation of at least some of the TrkA by Gö6976 instead of K252a.

3.3.2 Mechanism for K252a promotion of survival in the absence of NGF signaling

This study has not identified the mechanism by which high concentrations of K252a supports survival when applied to either the distal axons or the cell bodies/proximal axons of NGF-deprived neurons. Possible mechanisms include the activation of a signaling molecule by K252a that promotes survival or inhibition of a signaling molecule that promotes apoptosis. There is no reason to exclude the possibility that K252a promotes survival by acting on multiple targets, thereby activating more than one mechanism. A potential mechanism of action for K252a proposed by a previous study is Src-dependent activation of Akt by K252a, as demonstrated in PC12 cells (Roux et al., 2002). This study also provided evidence that K252a treatment activated Akt in sympathetic neurons suggesting that K252a could promote survival through Akt-mediated signaling. However, in contrast to this study, MacInnis et al. (2003) did not observe activation of Akt in K252a-treated sympathetic neurons at any concentration tested (range = 100-1000 nM), which suggested that K252a can also promote survival by alternative mechanism(s). Another set of targets for K252a is members of the mixed lineage kinase (MLK) family (Hudkins et al., 2007; Roux et al., 2002). MLKs are implicated in promoting the apoptosis of sympathetic neurons following NGF deprivation (Xu et al., 2001). Therefore, it is possible that the survival-promoting effect of K252a may be due to its ability to inhibit the apoptotic pathway by acting on MLKs.

The ability of K252a to promote survival when applied to the distal axons of sympathetic neurons is intriguing, because it raises the possibility that K252a is capable of producing retrograde survival signals or, alternatively, that K252a can inhibit apoptotic signaling in the distal axons that negatively regulates survival. At the time this study was performed, there was no evidence that apoptotic signaling occurred in axons in response to NGF deprivation. However, the results presented in the next chapter of this thesis demonstrate that axon apoptotic signaling induced by NGF deprivation does exist in sympathetic neurons and plays a key role in promoting apoptosis.

3.3.3 Exploring alternative interpretations for the results obtained with Gö6976

The results of this study have been interpreted to indicate that Gö6976 inhibits TrkA phosphorylation that is necessary for TrkA's activity. Thus, experiments where Gö6976 is used to inhibit TrkA in the cell bodies/proximal axons demonstrate that TrkA activity is not required in the cell bodies/proximal axons for survival mediated by NGF retrograde signals. However, there are potential alternative explanations for the results obtained with Gö6976 that should be discussed before drawing this conclusion.

One potential explanation is that Gö6976 does not completely inhibit TrkA activity. Results of this study demonstrated that application of Gö6976 to distal axons supplied with NGF completely prevented the ability of NGF to produce retrograde signals, presumably via TrkA, which were required for survival. However, is it possible that TrkA inhibited by Gö6976 still retains some residual functions, which are sufficient once TrkA arrives at the cell bodies/proximal axons to mediate downstream signaling that supports survival? The activation of TrkA is associated with its phosphorylation at tyrosine residues. In this study, it was demonstrated that Gö6976 effectively inhibited the phosphorylation of TrkA at residue Y490, which regulates a critical binding site for adaptor molecules implicated in facilitating downstream survival signaling. However, TrkA contains several other tyrosines that are phosphorylated upon activation and that also regulate survival signaling. These include phosphorylation of tyrosines in the kinase domain of TrkA that function to promote its kinase activity. The effect of Gö6976 on the phosphorylation of these tyrosines was not examined in this study. However, the original study analyzing the ability of Gö6976 to inhibit TrkA demonstrated that Gö6976 treatment completely prevented the tyrosine phosphorylation of TrkA induced by NGF (Behrens et al., 1999). This suggests that all tyrosine phosphorylation of TrkA, including sites that contribute to kinase activity, are inhibited by Gö6976 treatment. The ability of Gö6976 to completely inhibit Trk phosphorylation makes sense considering that Gö6976 likely acts on TrkA by occupying its ATP binding pocket, which would prevent TrkA from carrying out its kinase function required for both autophosphorylation and downstream signaling. Therefore, it is highly unlikely that TrkA inhibited by Gö6976 is able to participate in any signaling, including signaling necessary for promoting survival.

Another issue that can be raised about the results obtained with Gö6976 in this study involves the kinetics of TrkA inhibition of Gö6976. The earliest time point at which inhibition of TrkA phosphorylation by Gö6976 was measured is 1 hour and a dramatic reduction of TrkA phosphorylation was observed at that time in response to Gö6976 treatment. However, the minimum time required for Gö6976 to terminate the phosphorylation of TrkA was not determined in this study, which leaves the possibility that phosphorylated TrkA exposed to Gö6976 may continue to signal for an undetermined period of time, prior to deactivation. A scenario can be proposed where a retrograde survival signal requiring phosphorylated TrkA encountering Gö6976 travels through the proximal axons that could span a length of up to 1 mm, arrives at the cell bodies, and is able to participate in enough downstream signaling prior to its deactivation to effectively mediate survival. Experiments in this study showed no significant increase in TrkA phosphorylation in the cell bodies/proximal axons compared to control cultures completely deprived of NGF indicating that if there is some TrkA phosphorylation due to a lag in deactivation by Gö6976, the amount is undetectable. The fact that there is no diminished capacity for NGF supplied to distal axons to support survival even when all detectable TrkA phosphorylation in the cell bodies/proximal axons associated with NGF signaling is blocked by Gö6976 argues strongly against a critical role for TrkA phosphorylation in the cell bodies in mediating a retrograde survival signaling.

Gö6976 was originally characterized for its ability to inhibit conventional members of the protein kinase C (PKC) family (IC_{50} = 2.9 nM for PKCα, IC_{50} = 6.2 nM for PKCβ1; Martiny-Baron et al., 1993). Since Gö6976 was used at concentrations ranging from 1-25 nM in this study, it is plausible that PKCs were inhibited by Gö6976 in the experiments performed. This raises the issue of whether the results observed with Gö6976 could somehow be explained by a PKC-dependent mechanism. For example, it could be hypothesized that Gö6976's inhibition of PKC in the cell bodies/proximal axons generates a survival signal that compensates for the loss of retrograde survival signals caused by Gö6976's concurrent inhibition of TrkA. If inhibition of PKC does generate a survival signal, then it might be expected that this signal would also operate in the absence of NGF retrograde signaling. However, in this study it was demonstrated that supplying Gö6976 to the cell bodies/proximal axons in NGF-deprived cultures did not support survival. Therefore, in cultures generating NGF retrograde signals from the distal axons, the complete support of survival observed when Gö6976 was supplied to the cell bodies/proximal axons is not due to the generation of a survival signal caused by the inhibition of PKC. However, to completely rule out the possibility that PKC inhibition contributes to the results of this study, the experiments could be repeated with Trk inhibitors that do not inhibit PKC. Recently, a cell-permeable compound synthesized by modification of staurosporine has been shown to have greater selectivity, *in vitro*, for TrkA ($IC_{50} = 8$ nM) over PKC ($IC_{50} = 4$ μM) (Tripathy et al., 2008).

3.3.4 Possible mechanisms for an NGF retrograde signal that does not require phosphorylation of TrkA

All NGF retrograde survival signals are likely to share certain properties that allow them to effectively carry out their function. For example, retrograde signals are probably initially activated in axons as a result of local NGF signaling. The retrograde survival signals generated must possess the ability to retrogradely transmit that signal to the cell bodies. The signals must be delivered to the cell bodies in amounts that can effectively facilitate the downstream signaling necessary for supporting survival.

A retrograde survival signal that has been predicted by the signaling endosome hypothesis is composed of endosomes carrying both NGF and phosphorylated TrkA. However, since experiments in this study demonstrate that survival can be mediated by retrograde signals that do not require the phosphorylation of TrkA in the proximal axons or the cell bodies, such retrograde signals must use other molecules to carry their signal to the cell bodies and mediate downstream survival signaling. Stimulation of axons with NGF results in the local activation of signaling molecules downstream of TrkA, and it is plausible that some may function as carriers for retrograde survival signals. In sympathetic neurons, PI3K (Kuruvilla et al., 2000), Akt (Kuruvilla et al., 2000), and Erk1/2 (MacInnis et al., 2003) are activated in axons by NGF. In sensory neurons, NGF stimulation of axons results in the local activation of Erk 1/2, p38 (Delcroix et al., 2003), Erk 5 (Watson et al., 2001), and local translation of CREB (Cox et al., 2008).

Given the results of this study which demonstrate that NGF retrograde signals that do not require TrkA phosphorylation efficiently induce the phosphorylation of Akt and CREB in the cell bodies/proximal axons, it is likely that some retrograde survival signals may be composed of members of the pathways associated with activating these molecules. One potential candidate is PI3K, which is activated in axons (Kuruvilla et al., 2000) and shows retrograde movement in the rat sciatic nerve both *in vivo* (Johanson et al., 1995) and in *ex vivo* preparations (Delcroix et al., 2003). PI3K delivered to the cell bodies would be able to facilitate the activation of Akt, which could then induce downstream CREB activation. Another potential candidate for carrying a retrograde survival signal is Akt itself. The results from this study and previous studies (Kuruvilla et al., 2000; MacInnis et al., 2003; Ye et al., 2003) have shown that Akt is phosphorylated in axons of sympathetic neurons in response to NGF. Delcroix et al. (2003) showed retrograde accumulation of activated Akt in *ex vivo* preparations of the rat sciatic nerve in response to NGF, suggesting that Akt, like PI3K, moves retrogradely. Therefore, retrograde movement of Akt functioning as a survival signal could account for the appearance of phosphorylated Akt in the cell bodies, which would then lead to the downstream activation of CREB. Another retrograde survival signal that may be relevant to our results has been demonstrated in sensory neurons, where NGF-induced axon translation of CREB followed by its retrograde transport to the cell bodies is required for CREB transcription activity and survival (Cox et al., 2008). However, according to the results of this study, the phosphorylation of CREB at Ser133 associated with its activation is only detected in the cell bodies/proximal axons and not in the distal axons. Therefore, CREB is not initially carried in its active form, and it is likely that other retrograde survival signals are needed to induce its activation, perhaps when the CREB reaches the cell bodies. Thus, although retrograde transport of axon-translated CREB may be important for survival, it probably requires other retrograde survival signals to carry out its function.

How would the retrograde survival signals such as the ones proposed above be retrogradely transmitted? It is possible that activated signaling molecules could be transported by motors retrogradely moving along microtubules. This would require that they would have some mechanism by which to link to the retrograde motors. One possibility is that the molecules are able to associate with membrane-based cargo such as vesicles or endosomes that are being retrogradely transported. Indeed there is evidence that endosome fractions isolated from *ex vivo* rat sciatic nerve contain, along with NGF and TrkA, several downstream signaling molecules including Gab2, Rap1, B-Raf, MEK1/2, Erk1/2, p38, and PI3K (Delcroix et al., 2003). These results suggest that these molecules are somehow tethered to endosomes, possibly through their recruitment to the membrane following TrkA activation. Signaling molecules such as PI3K and Akt may maintain association with TrkA containing endosomes through scaffolding proteins (Figure 3-6A) such as APPL1 (Adaptor protein, phosphotyrosine interaction, PH domain and leucine zipper containing 1). APPL1, along with being able to bind Akt and subunits of PI3K (Mitsuuchi et al., 1999), is also able to bind TrkA directly and indirectly via interaction with GIPC (Lin et al., 2006; Varsano et al., 2006). Moreover, APPL1 is present in the axons of sympathetic neurons and is found in endosome fractions from

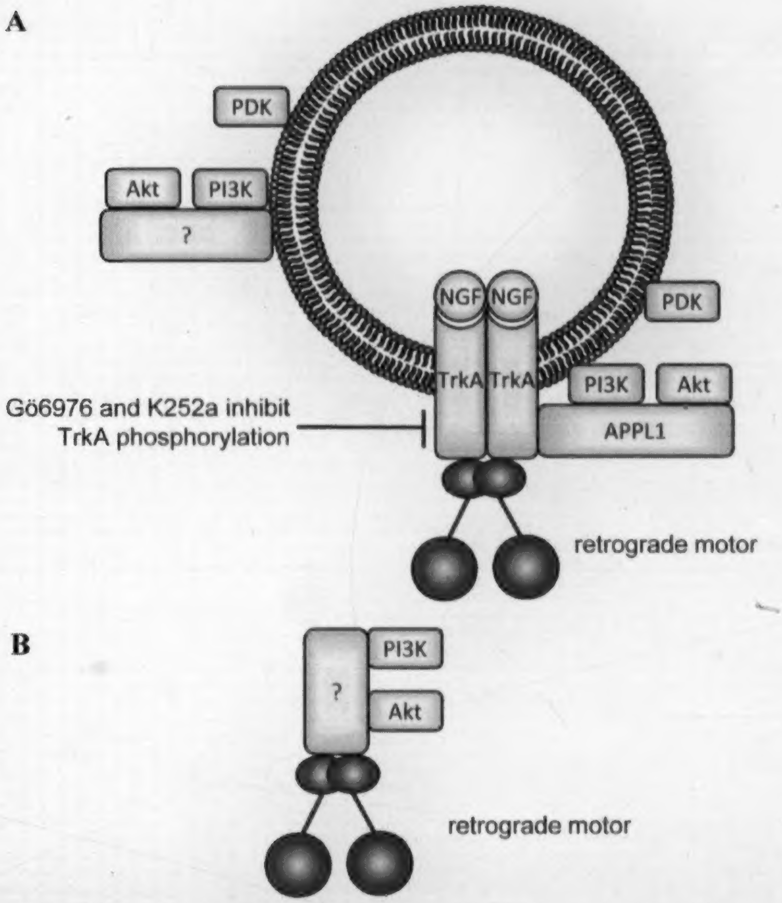


Figure 3-6. Examples of potential retrograde survival signals not requiring the retrograde transport of phosphorylated TrkA. A) Survival signals carried by retrogradely transported endosomes. Signaling molecules activated downstream of TrkA in axons may be initially recruited to membranes by TrkA-associated adaptor proteins which are internalized and trafficked into endosomes. TrkA may be able to directly link endosome cargo to the retrograde motor, dynein. The survival signals must remain associated with endosomes even when TrkA phosphorylation is inhibited by compounds such as Gö6976 and K252a. This could be achieved by scaffold proteins such as APPL1 that can associate with PI3K, Akt, and TrkA regardless of the phosphorylation state of TrkA. Signaling molecules such as PI3K and Akt could also be tethered to endosomes without TrkA by scaffold proteins (?) that have not been identified yet. B) Survival signals may also be able to link with dynein motors without endosomes via an unidentified linker protein (?). Note that PI3K and Akt have been used as examples of potential retrograde survival signals in this diagram but other signaling molecules downstream of TrkA may also function as retrograde survival signals that are retrogradely transported by the mechanisms described above.

PC12 cells, some of which contained phosphorylated TrkA (Lin et al., 2006). Therefore, it is plausible that retrograde survival signals downstream of TrkA could be carried by association with retrogradely transported endosomes containing TrkA. However, in order for such a mechanism to be consistent with the results obtained in this study, the signaling molecules, although initially recruited to endosomes by phosphorylated TrkA, could not be dependent on TrkA phosphorylation for their continued association with retrogradely transported membrane cargo since TrkA phosphorylation is not required for the effective transmission of survival signals as they travel through the proximal axons prior to arriving at the cell bodies. The finding that APPL1 can directly bind TrkA even when Trk phosphorylation is inhibited by K252a (Lin et al., 2006) offers a means by which activated PI3K and Akt could continue to associate with retrogradely transported endosomes containing dephosphorylated TrkA (Figure 3-6A).

Although there is some evidence to support a retrograde transmission mechanism involving TrkA-containing endosomes, several other mechanisms can also be proposed. For example, signaling molecules could be retrogradely transported by tethering to endosomes without TrkA (Figure 3-6A). Signaling molecules may also be able to directly link to retrograde motors to mediate their transport (Figure 3-6B). Previous reviews have also proposed that transmission of signals could be achieved by lateral propagation of the phosphorylation of kinases or propagation of calcium waves (Campenot and MacInnis, 2004; Ginty and Segal, 2002; Senger and Campenot, 1997). Given what is known about retrograde survival signals presently, and the possibility that multiple signals may be contributing to neuronal survival, it would be premature to exclude any of these mechanisms at this time.

One issue that is often raised regarding retrograde survival signals is how they remain activated as they travel long distances to the cell bodies. One attractive feature of the signal predicted by the signaling endosome hypothesis is that the NGF trapped in the signaling endosome would be available to reactivate the endosomal TrkA for the duration of the retrograde journey, in the event that the TrkA became deactivated by phosphatases. It could be hypothesized that retrograde survival signals composed of other signaling molecules may not be afforded the same protection. Phosphatases for PI3K and Akt are present in axons, and their activity is implicated in regulating axon growth by acting on PI3K and Akt signaling at growth cones (Chadborn et al., 2006; Ooms et al., 2006). However, it has not been determined if phosphatases are constitutively activated throughout the axon or that they effectively deactivate retrogradely moving signaling molecules before they can arrive at their cell body targets. Therefore, there is no reason to assume that retrograde survival signals composed of signaling molecules such as kinases are not capable of retaining their activation during their retrograde transmission to the cell bodies.

3.3.5 Rationale for having multiple retrograde signaling mechanisms contributing to survival

The results of this study convincingly establish that NGF can fully support the survival of sympathetic neurons using retrograde signals that do not require the phosphorylation of TrkA in the cell bodies/or proximal axons. The findings are entirely consistent with the conclusions of MacInnis et al. (2003) using the Trk inhibitor, K252a. However, these results should not be interpreted to mean that retrograde survival signals that do utilize phosphorylated TrkA may not also be capable of contributing to the survival of sympathetic neurons. Indeed, in the study by Ye et al. (2003), NGF retrograde survival of sympathetic neurons seems to be critically dependent on signals requiring the retrograde transport of NGF and phosphorylated TrkA. As discussed in the Introduction (see Section 1.10.10), it is possible that the specific culturing parameters used in Ye et al. (2003) may have enhanced the dependence of the sympathetic neurons for a retrograde signal requiring phosphorylated TrkA in the cell bodies, a dependence that was not observed in the present study or MacInnis et al. (2003) because other retrograde signals were capable of compensating for its absence.

It is completely reasonable to hypothesize that NGF is capable of producing multiple retrograde signals that promote survival. Having a range of retrograde survival signals may allow for the neuron to recognize and respond appropriately to a wider variety of conditions. Thus, each retrograde signal may be optimized to operate under specific parameters. For instance, it is possible that some retrograde survival signals require higher concentrations of NGF for activation than other retrograde survival signals. It is also possible that some retrograde signals have greater longevity; they are capable of retaining their activation over longer distances through the axon or after arriving at the cell body. Since each retrograde survival signal would be composed of different signaling molecules, it is reasonable to think that each survival signal may activate a different range of downstream signals. Therefore, some survival signals may promote survival more effectively than others because they activate the more critical survival pathways or because they can activate multiple survival pathways. It is also plausible that some retrograde signals may have other functions besides promoting survival, such as facilitating the competition for survival between neurons. A retrograde survival signal requiring retrograde transport of NGF would, as a consequence, diminish the amount of NGF available at the target area, thereby making it more difficult for neighboring neurons to obtain enough NGF signaling to promote their survival.

Another possible reason for the operation of multiple retrograde survival signals is that one signal may simply not be enough to support survival under challenging conditions that may be encountered *in vivo*. For example, when the axon of a neuron is initially arriving at its target, there will be relatively few axon terminals available to bind NGF. During this period of initial innervation, the amount of NGF and phosphorylated TrkA that can be internalized into endosomes to serve as a retrograde survival signal may simply not generate enough signaling on its own to support survival. Thus, activation of additional retrograde survival signals may be required in order to amplify signaling to a level that is sufficient to support survival.

3.3.6 Potential implication of results in understanding survival regulation by other neurotrophic factors during development and in adulthood

Many neurons depend on target-derived factors for their survival during development. The most well characterized of these factors are neurotrophins, which critically regulate the survival of several populations of sympathetic and sensory neurons of the peripheral nervous system (for review see Huang and Reichardt, 2001). However, other neurotrophic factors are also beginning to be recognized for their ability to promote target-dependent survival of specific neuron populations. For example, members of the glial cell line-derived neurotrophic factor (GDNF) family have been implicated in promoting target-dependent survival of populations of parasympathetic neurons (Lahteenmaki et al., 2007), dopamine neurons (Burke, 2006), and motor neurons

(Henderson et al., 1994; Oppenheim et al., 1995; Whitehead et al., 2005). Other neurotrophic factors such as cardiotrophin-like cytokine (CLC), cytokine-like factor 1 (CLF) (Forger et al., 2003), and hepatocyte growth factor (Yamamoto et al., 1997) have also been found to regulate the survival of specific populations of motor neurons. Since neurotrophic factors share a common ability to induce downstream singling events through binding to cognate receptors (Airaksinen and Saarma, 2002; Davis et al., 1993; Reichardt, 2006), it is plausible that other neurotrophic factors function in a manner similar to NGF to produce retrograde survival signals that promote survival. Several of the neurotrophic factors such as neurotrophins (Kuruvilla et al., 2004; Watson et al., 2001) and GDNF (Coulpier and Ibanez, 2004) have been shown to induce signaling in axons, raising the possibility that some of the activated molecules may be utilized as retrograde signals that contribute to developmental survival. Thus, it is important to continue to study all potential retrograde survival signals as it may lead to a better understanding of the complex process of nervous system development.

There is also evidence that neurotrophins continue to regulate survival in adulthood. Diminished neurotrophic factor signaling has been implicated as a contributing factor for the neurodegeneration associated with diseases such as Alzheimer's disease, Huntington's disease, and Parkinson's disease (reviewed by Fumagalli et al., 2008; Williams et al., 2006). Application of neurotrophic factors has been shown to offer neuroprotection to affected neurons in cell culture and animal models of these diseases (reviewed by Fumagalli et al., 2008). Based on these observations, treatments are being developed that involve the targeted delivery of exogenous neurotrophic factors to degenerating neurons (Bloch et al., 2004; Gasmi et al., 2007;

Tuszynski et al., 2005). Therefore, there is a potential clinical relevance for understanding the mechanisms underlying retrograde signals, since such knowledge could be useful for developing strategies that enhance the retrograde signaling induced by neurotrophin treatment or, alternatively, that produce retrograde signaling without neurotrophins.

CHAPTER 4:

THE AXON APOPTOTIC SIGNAL

A version of this chapter has been published.
Mok, S.A., K. Lund, and R.B. Campenot. 2009. A retrograde apoptotic signal originating in NGF-deprived distal axons of rat sympathetic neurons in compartmented cultures. *Cell Res*. 19(5):546-60. K. Lund contributed Figure 4-2A and the representative experiment shown in Figure 4-8.

4.1 Rationale

The neurotrophic factor hypothesis predicts that, *in vivo,* neurons that have extended axons to their target of innervation must then compete for limiting amounts of the target-secreted neurotrophins that promote their survival (Levi-Montalcini and Angeletti, 1968). Neurons that are able to procure enough signaling by neurotrophins survive, whereas those that fail to obtain sufficient neurotrophin signals undergo apoptosis. All current mechanisms that have been proposed to explain how neurotrophins such as NGF promote survival *in vivo* share the general principle that NGF acting on axons generates survival signals that are then retrogradely transmitted to the cell bodies to promote survival (Campenot and MacInnis, 2004; Howe and Mobley, 2004; Zweifel et al., 2005). In this study, evidence is provided for a fundamentally different mechanism of NGF retrograde survival signaling. In this mechanism, NGF acting locally on axons, promotes the survival of sympathetic neurons by suppressing the generation of a retrograde apoptotic signal. Loss of NGF signaling in axons results in the production of the apoptotic signal and its retrograde transmission to the cell bodies where it induces the activation of a critical apoptotic signal event, *c*-jun phosphorylation. Moreover, blocking the axon apoptotic signal by applying various inhibitor compounds to the axons prevented the apoptosis normally induced by NGF deprivation. This suggests that the axon apoptotic signal is a key factor regulating the survival of sympathetic neurons. The discovery of the axon apoptotic signal reveals that the regulation of sympathetic neuron survival is more complex than previously appreciated and may lead to valuable insights into the understanding of not only nervous system development but also other apoptotic processes associated with neurotrauma or neurodegenerative diseases.

4.2 Results-Initial evidence for an axon apoptotic signal activated during NGF deprivation

4.2.1 The activation of the pro-apoptotic transcription factor, *c*-jun, in cell bodies is controlled by NGF signaling in the distal axons

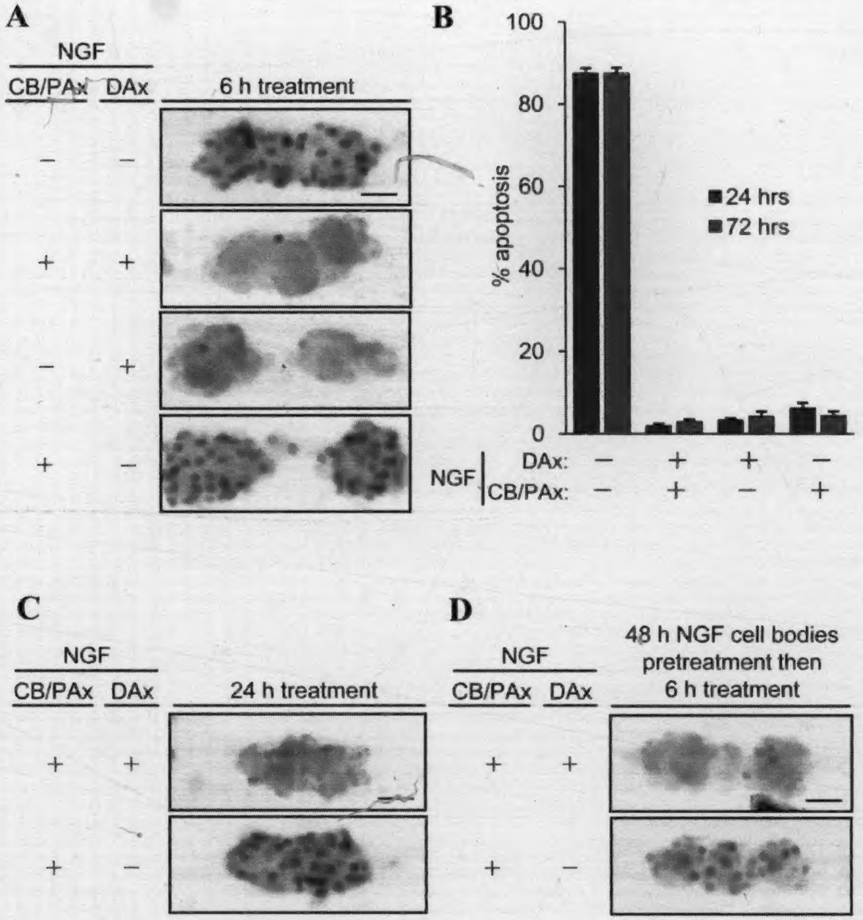
Current mechanisms for how NGF signaling from axons supports survival all involve the generation and delivery of retrograde survival signals to the cell bodies where they promote local downstream survival signaling that prevents the induction of apoptosis. The first experiment of this study set out to test the hypothesis that apoptosis is initiated by the loss of survival signaling at the cell bodies.

To assay for initiation of the apoptotic program in sympathetic neurons the activation state of the transcription factor, *c*-jun, was monitored. Activation of *c*-jun was chosen because it has been well characterized as an early event in response to NGF deprivation (Eilers et al., 1998; Ham et al., 2000; Virdee et al., 1997), and deletion of *c*-jun from sympathetic neurons prevents apoptosis caused by NGF deprivation (Palmada et al., 2002), suggesting that *c*-jun is essential for the apoptotic program. The transcription promoting activity of *c*-jun is stimulated by phosphorylation at residues Ser63 and Ser73 (Pulverer et al., 1991; Smeal et al., 1991). Phosphorylated *c*-jun must translocate to the nucleus (Waldmann et al., 2007) in order to promote changes in gene expression. To test if survival signaling at the cell bodies was sufficient to prevent *c*-jun activation, compartmented cultured sympathetic neurons were supplied with NGF on the distal axons (DAx) and/or the cell bodies/proximal axons (CB/PAx). A group of control cultures were not supplied with any NGF. In all the experiments of this study, medium for compartments that were not supplied with NGF contained 24 nM NGF function-blocking antibodies to prevent any residual NGF in the compartment from signaling. The cultures were treated for 6 h with the various NGF regimes then fixed and immunohistochemically stained for phosphorylated *c*-jun (Ser63).

Consistent with the hypothesis that loss of survival signals leads to apoptotic induction, compartmented cultures that were not supplied with NGF in any compartment contained many cell bodies that positively stained for nuclear localized, phosphorylated, *c*-jun (Figure 4-1A). Cultures supplied with NGF in all compartments or only in the distal axons compartments displayed almost no incidence of nuclear phosphorylated *c*-jun. These results are also consistent with the hypothesis, since cultures supplied with NGF on the distal axons are presumed to initiate survival signals that are retrogradely transmitted to the cell body to mediate downstream survival signaling. The lack of *c*-jun phosphorylation observed in these groups correlated with the prevention of apoptosis. Cultures supplied with NGF only at the distal axons or in all compartments underwent virtually no apoptosis after 72 h (3% and 4%, respectively) compared to the no NGF controls (87%) (Figure 4-1B).

The effect of supplying NGF to the cell bodies/proximal axons but not to the distal axons was also tested. Under these conditions, it was expected that NGF would effectively initiate signaling through TrkA receptors directly at the cell bodies, resulting in full support of neuronal survival. Indeed, when neurons were supplied with NGF only at their cell bodies/proximal axons, only 4% apoptosis was observed at 72 h (Figure 4-1B). Therefore, it was completely unexpected when cultures supplied with NGF at their

Figure 4-1. Sustained activation of the pro-apoptotic molecule, *c*-jun, is induced by local NGF withdrawal at the distal axons even when NGF survival signals are supplied directly to the cell bodies. A) *c*-jun is activated when NGF is removed from distal axons. Distal axons (DAx) and cell bodies/proximal axons (CB/PAx) were supplied with 50 ng/ml NGF (+) or medium lacking NGF and containing NGF antibodies (–) as indicated for 6 h. Following treatment, CB/PAx were processed for immunocytochemistry with an antibody recognizing phosphorylated *c*-jun (Ser63). Activation of *c*-jun is characterized by nuclear accumulation of phosphorylated *c*-jun. The scale bar equals 50 µm. Results are representative of 3 independent experiments. B) Local withdrawal of NGF from distal axons does not result in apoptosis if NGF is provided to the cell bodies/proximal axons. NGF (+) or NGF antibodies (–) were supplied in compartments as indicated for 24 h (blue bars) or 72 h (red bars). Apoptosis of a neuron was defined by the occurrence of nuclear condensation and fragmentation and was graphed as the mean percentage of apoptosed neurons per group. At 72 h, neurons lacking nuclear staining were also included in the count (see Section 2.3 for details). A minimum of 250 neurons were assessed per culture. Data were compiled from two experiments (± SEM, n = 6 cultures). (C and D) Cultures were treated and analyzed as in A). Results are representative of 3 independent experiments. C) Activation of *c*-jun following 24 h of treatment. D) Cultures were pretreated for 48 h with 50 ng/ml NGF in all compartments prior to the experimental treatment.


A
NGF
CB/PAx
DAx
6 h treatment
− −
+ +
− +
+ −
B
100
80
60
40
20
0
% apoptosis
■24 hrs
■72 hrs
NGF
DAx: − + + −
CB/PAx: − + − +
C
NGF
CB/PAx
DAx
24 h treatment
+ +
+ −
D
NGF
CB/PAx
DAx
48 h NGF cell bodies pretreatment then 6 h treatment
+ +
+ −

cell bodies/proximal axons but not at their distal axons displayed robust staining for nuclear, phosphorylated *c*-jun, similar to cultures completely deprived of NGF (Figure 4-1A). The presence of this nuclear, phosphorylated *c*-jun signal persisted even after 24 h of treatment (Figure 4-1C). To ensure that the cell bodies were generating strong survival signaling at the onset of the experiment, neurons were pretreated with 50 ng/ml NGF for 48 h in all compartments. Pretreated cultures supplied with NGF only at the cell bodies/proximal axons for 6 h still displayed nuclear accumulation of phosphorylated *c*-jun (Figure 4-1D).

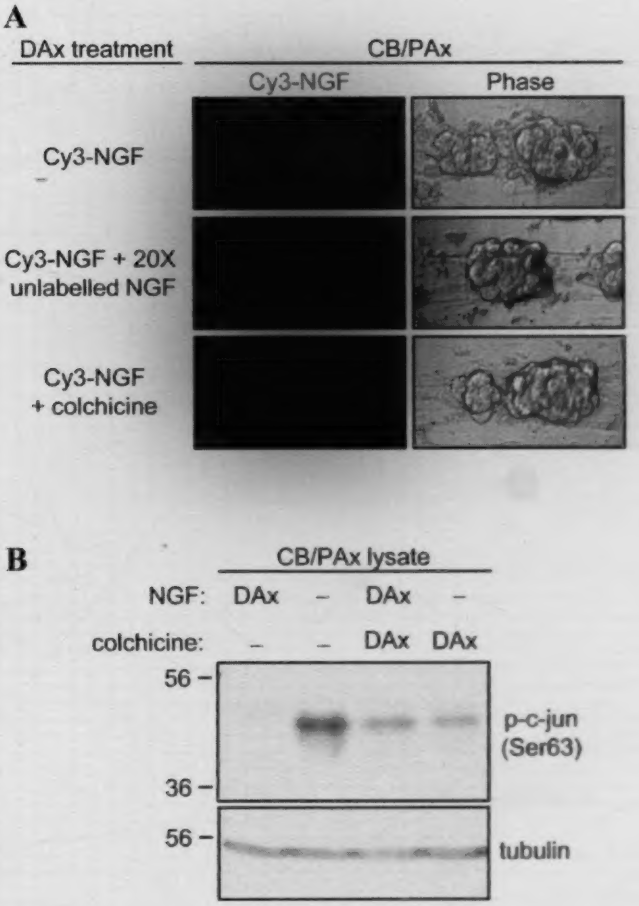
Surprisingly, the results of the experiment showed that *c*-jun activation did not correlate with the loss of NGF survival signals at the cell bodies/proximal axons. Instead what was discovered was that *c*-jun activation only occurred under conditions where NGF had been withdrawn from the distal axons. Based on these data it is hypothesized that the activation of the pro-apoptotic molecule, *c*-jun, is suppressed by NGF signaling at the distal axons. Loss of NGF signaling in the distal axons generates an axon apoptotic signal that travels retrogradely to the cell bodies to induce *c*-jun activation. Since *c*-jun activation is required for the apoptosis of sympathetic neurons in response to withdrawal of NGF (Palmada et al., 2002), this suggests that the axon apoptotic signal responsible for its activation is also required for apoptosis.

Interestingly, survival signaling provided by NGF supplied directly to the cell bodies/proximal axons is unable to deactivate the axon apoptotic signal that leads to *c*-jun activation, which indicates that NGF signaling in distal axons and NGF signaling in the cell bodies/proximal axons are not equivalent. Since the data show that neurons receiving NGF at the cell bodies/proximal axons do not undergo apoptosis, NGF signaling initiated at the cell bodies/proximal axons must support survival by counteracting the actions of the axon apoptotic signal at an event downstream of *c*-jun or by a *c*-jun-independent pathway.

4.2.2 The axon apoptotic signal requires microtubule-based transport in distal axons to activate *c*-jun

If there is an apoptotic signal originating from the distal axons, then it is expected that the signal needs to be retrogradely transmitted to the cell bodies in order to induce nuclear accumulation of phosphorylated *c*-jun. One potential mode of transmission is via linkage to retrogradely transported motors traveling along microtubules. This mode of transport can be blocked by destabilizing the microtubules with the microtubule depolymerizing agent, colchicine. To assay if colchicine disrupted microtubule-based transport, the retrograde transport of NGF, which has been previously shown to be microtubule-dependent (Hendry et al., 1974), was used as an indicator. To visualize the movement of NGF, it was conjugated to fluorescent Cy3 molecules (Cy3-NGF). Cy3-NGF (50 ng/ml) was supplied only to the distal axons, and retrograde transport was indicated by the accumulation of the Cy3-NGF in the cell bodies located in the cell bodies/proximal axon compartment. Following 6 h of treatment with Cy3-NGF at the distal axons, a strong signal for Cy3-NGF was detected in the cell bodies (Figure 4-2A). The retrograde transport of Cy3-NGF could be competed out by addition of a 20-fold excess of unlabeled NGF to the distal axons, suggesting that the process was receptor-mediated and not due to non-specific uptake and transport of Cy3-NGF. Application of 10 μM colchicine to the distal axons was sufficient to prevent the retrograde accumulation of Cy3-NGF in the cell bodies, indicating disruption of the microtubule

Figure 4-2. Colchicine applied to distal axons during NGF withdrawal blocks activation of *c*-jun in the cell bodies. A) Colchicine blocks retrograde transport of Cy3-labelled NGF (Cy3-NGF). Cy3-NGF (50 ng/ml) was applied to the distal axons (DAx). In addition to the Cy3-NGF, 20-fold excess unlabelled NGF (1 μg/ml) or colchicine (10 μM) was supplied to the DAx as indicated. Cell bodies/proximal axons (CB/PAx) were analyzed by fluorescence imaging for the accumulation of Cy3-labelled NGF after 6 h of treatment. Results are representative of 3 independent experiments. B) Colchicine applied to distal axons blocks *c*-jun activation during NGF withdrawal. Distal axons (DAx) were supplied with 50 ng/ml NGF or NGF antibodies (–) in the absence or presence of 10 μM colchicine where indicated. Cell bodies/proximal axons in all cultures were treated with NGF antibodies. Following a 6 h treatment, cell bodies/proximal axon lysates were analyzed by immunoblotting with an antibody recognizing phosphorylated *c*-jun (Ser63). The blot was reprobed for actin as a loading control. Results are representative of 4 independent experiments.


A
DAx treatment
CB/PAx
Cy3-NGF
Phase
Cy3-NGF
Cy3-NGF + 20X unlabelled NGF
Cy3-NGF + colchicine
B
CB/PAx lysate
NGF: DAx – DAx –
colchicine: – – DAx DAx
56
36
56
p-c-jun (Ser63)
tubulin

system required for the retrograde transport of molecules such as NGF. It was next assayed if destabilizing microtubules with colchicine also prevented the axon apoptotic signal from activating *c*-jun by blocking the retrograde transmission of the signal to the cell bodies. The distal axons of cultures were either supplied with NGF to suppress the axon apoptotic signal or without NGF to induce the axon apoptotic signal. The cell bodies proximal/axons in all groups did not receive NGF. Colchicine (10 μM) was supplied to the distal axons in the indicated treatment groups for the duration of the experiment. The cell bodies/proximal axons were harvested after 6 h of treatment and analyzed by immunoblot. Cultures not supplied with any NGF (a condition from here on called NGF deprivation) showed a strong signal for phosphorylated *c*-jun (Ser63) in their cell bodies/proximal axons that was not detected in cultures receiving NGF at their distal axons (Figure 4-2B). Application of 10 μM colchicine to the distal axons in cultures undergoing NGF deprivation greatly reduced the intensity of the signal for phosphorylated *c*-jun. This result is consistent with the hypothesis that *c*-jun activation is mediated by the delivery of a retrograde death signal from the distal axons. Furthermore, it suggests that the retrograde transmission is mediated by a microtubule-dependent transport. These results argue against the concept that *c*-jun activation is caused by the loss of survival signals being delivered to the cell bodies. If this was the case, then blocking retrograde transport with colchicine would not have prevented *c*-jun activation.

A low level of *c*-jun phosphorylation was observed in colchicine-treated neurons regardless if the neurons were supplied with NGF or without NGF, suggesting that the small amount of phosphorylation may be induced as a side effect of colchicine application.

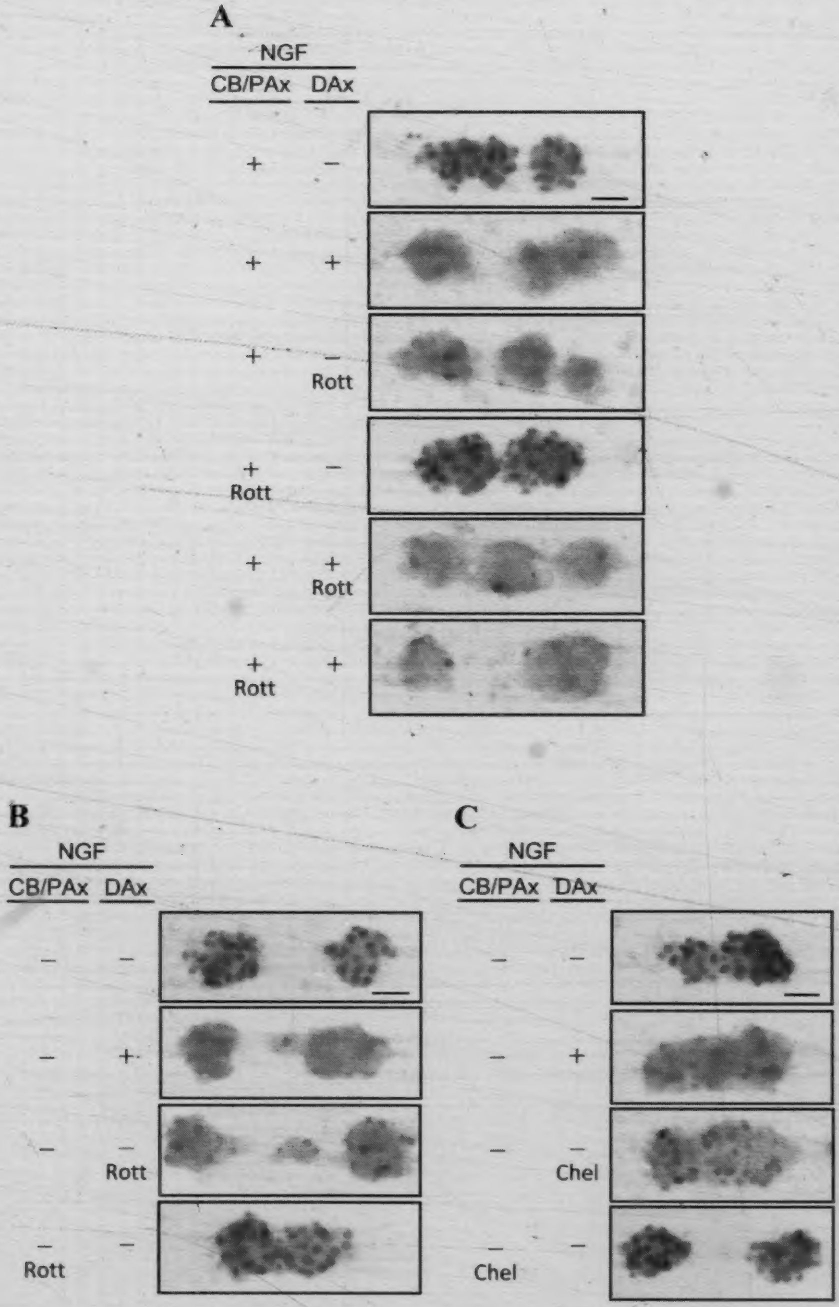
An attempt was made to determine if blocking the retrograde transport of the apoptotic signal with colchicine prevented apoptosis caused by NGF deprivation. Unfortunately, colchicine application to the distal axons resulted in the degeneration of the axons beginning at 12 h. Therefore, experiments that measured apoptosis completion occurring at 24 h were not feasible with colchicine-treated neurons.

4.2.3 The pharmacological agents, rottlerin and chelerythrine, prevent *c*-jun activation only when applied to distal axons

Two compounds, rottlerin and chelerythrine, were identified as having anti-apoptotic effects when applied to the distal axons but not when applied to the cell bodies/proximal axons (results described in Section 4.2.4). The discovery of the axon apoptotic signal led to the hypothesis that these inhibitors might elicit their effects by blocking this signal. Rottlerin was originally characterized as a PKC inhibitor, with specificity for the PKCδ isoform (IC_{50} = 3-6 μM in rat brain; Gschwendt et al., 1994). Similarly, chelerythrine was originally identified as a PKC inhibitor of conventional and novel PKC isoforms (Herbert et al., 1990).

The state of *c*-jun phosphorylation was used as an indicator to determine if rottlerin and chelerythrine blocked the axon apoptotic signal. The axon apoptotic signal was induced by supplying NGF to the cell bodies/proximal axons but not the distal axons for 6 h. A control group of cultures were supplied with NGF in all compartments. During the treatment, rottlerin (2.5 μM) was supplied to the distal axons or the cell bodies/proximal axons. The effect of chelerythrine was not assayed in this experiment. When distal axons were deprived of NGF, application of rottlerin to the distal axons during treatment blocked the appearance of nuclear, phosphorylated *c*-jun (Figure 4-3A).

Figure 4-3. Rottlerin and chelerythrine act exclusively on distal axons to prevent activation of pro-apoptotic *c*-jun. Cell bodies/proximal axons (CB/PAx) and distal axons (DAx) were supplied with 50 ng/ml NGF (+) or medium lacking NGF and containing NGF antibodies (–) as indicated for 6 h. For the duration of treatment, rottlerin (Rott, 2.5 μM) or chelerythrine (Chel, 10 μM) were also supplied to the DAx or CB/PAx as indicated. The cultures were then processed for detection of phosphorylated *c*-jun as in Figure 4-1A. The scale bar equals 50 μm. A) Rottlerin when applied to distal axons, but not when applied to cell bodies and proximal axons, prevented *c*-jun activation caused by local withdrawal of NGF from distal axons. Results are representative of 3 independent experiments. B) Rottlerin when applied to distal axons, but not when applied to cell bodies and proximal axons, prevented *c*-jun activation caused by complete withdrawal of NGF. Results are representative of 4 independent experiments. C) Chelerythrine prevents *c*-jun activation during NGF withdrawal only when applied to the distal axons. Results are representative of 3 independent experiments.


A
NGF
CB/PAx DAx
+ –
+ +
+ – Rott
+ Rott –
+ + Rott
+ Rott +
B
NGF
CB/PAx DAx
– –
– +
– – Rott
– Rott –
C
NGF
CB/PAx DAx
– –
– +
– – Chel
– Chel –

The level of nuclear staining was indistinguishable from the minimal staining observed in cultures supplied with NGF in all compartments. In contrast, application of rottlerin to the cell bodies/proximal axons did not prevent the phosphorylated *c*-jun signal. The *c*-jun activation observed was not caused by rottlerin application itself since rottlerin applied to the cell bodies/proximal axons did not result in *c*-jun activation when the cultures were supplied with NGF. These results suggest that rottlerin prevents *c*-jun activation by acting on a component of the axon apoptotic signal occurring specifically in the distal axons. Since rottlerin does not inhibit the effects of the apoptotic signal when supplied to the cell bodies/proximal axons, it indicates that rottlerin does not act on the molecules that carry the axon apoptotic signal to the cell bodies. Therefore, rottlerin is most likely functioning by inhibiting the initial generation of the retrograde apoptotic signal.

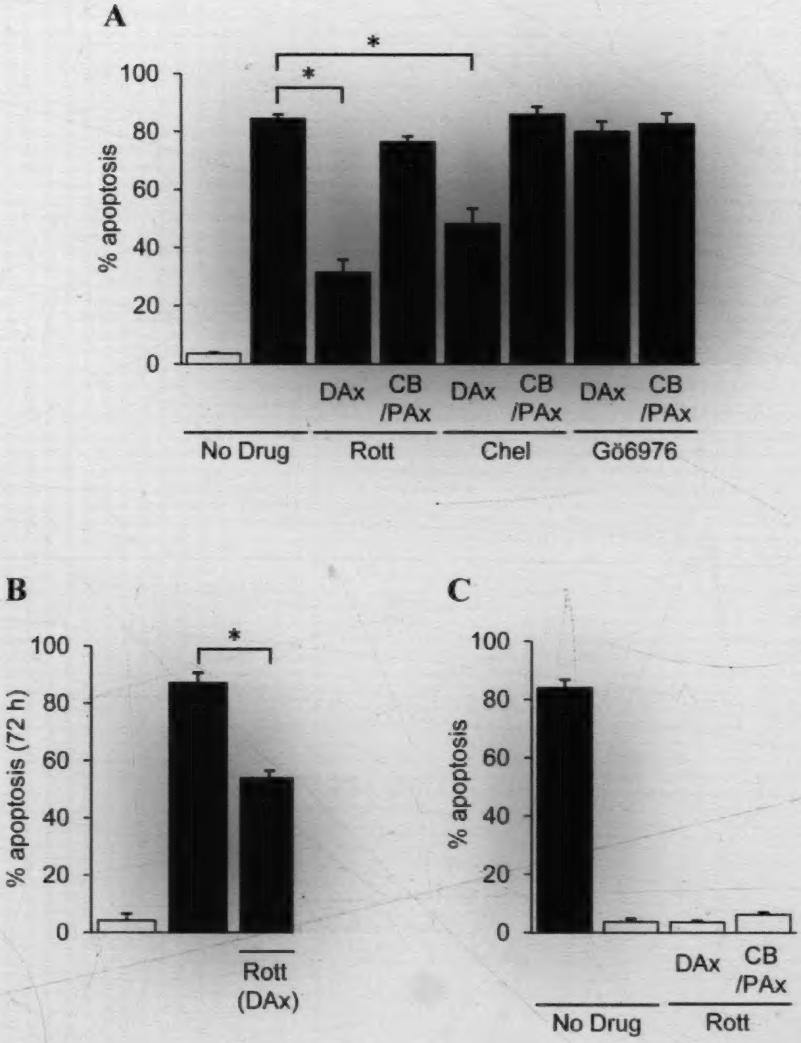
The ability of rottlerin and chelerythrine to block the axon apoptotic signal was next assessed in cultures completely deprived of NGF. NGF was supplied only to the distal axons or withdrawn from all compartments for 6 h in the absence or presence of 2.5 μM rottlerin at either the distal axons or cell bodies/proximal axons. The results followed the same pattern obtained in the preceding experiment. Only rottlerin application to the distal axons prevented nuclear accumulation of phosphorylated *c*-jun in NGF-deprived cultures (Figure 4-3B). A similar experiment was performed in which 10 μM chelerythrine was supplied to either the distal axons or the cell bodies/proximal axons during treatment. Only chelerythrine application to the distal axons diminished *c*-jun activation during NGF deprivation (Figure 4-3C). These results demonstrate that rottlerin and chelerythrine applied to distal axons prevents the induction of a key signaling event required for the apoptosis of sympathetic neurons deprived of NGF.

4.2.4 Rottlerin and chelerythrine prevent apoptosis only when applied to distal axons

Given that rottlerin and chelerythrine supplied to the distal axons blocked *c*-jun activation of neurons completely deprived of NGF and that *c*-jun is required for apoptosis in response to NGF deprivation (Palmada et al., 2002), it was expected that rottlerin and chelerythrine supplied to the distal axons would protect sympathetic neurons from apoptosis during NGF deprivation.

To confirm this, neurons were completely deprived of NGF for 24 h in the presence of inhibitors (rottlerin, chelerythrine, or Gö6976) supplied to the compartments indicated in Figure 4-4A. Apoptosis was indicated by condensation of the nucleus. In control cultures provided with NGF at their distal axons (white bar) only 4% apoptosis was observed (Figure 4-4A). When neurons were deprived of NGF (black bars) without drug, the rate of apoptosis increased to 84%. NGF deprivation of neurons in the presence of 2.5 μM rottlerin on the distal axons significantly decreased the apoptosis rate to 32%. When apoptosis was examined after 72 h of NGF deprivation (87% apoptosis), rottlerin supplied to the distal axons still significantly decreased apoptosis to 54% (Figure 4-4B). In contrast, application of rottlerin to the cell bodies/proximal axons during NGF deprivation did not provide any significant protection (76% apoptosis) even at 24 h (Figure 4-4A). The possibility that rottlerin application to the cell bodies/proximal axons caused apoptosis as a toxic side effect was examined by supplying rottlerin to cultures receiving NGF (Figure 4-4C). In cultures supplied with NGF at the distal axons, no significant increase in apoptosis was observed when rottlerin was applied to either the

Figure 4-4. Rottlerin and chelerythrine supplied to distal axons prevents apoptosis in response to NGF deprivation. Cell bodies/proximal axons (CB/PAx) of all cultures were given NGF antibodies, while distal axons (DAx) were treated with either 50 ng/ml NGF (white bars) or NGF antibodies (black bars). Concurrently, the PKC inhibitors, Rottlerin (Rott, 2.5 μM), Chelerythrine (Chel, 10 μM) or Gö6976 (25 nM) were supplied in the compartments indicated. Apoptosis was identified by condensed, fragmented nuclei. The percentage of apoptotic neurons per group is plotted (± SEM). A) Application of the PKCδ inhibitors, rottlerin and chelerythrine, to the distal axons but not to the cell bodies and proximal axons prevents apoptosis (data compiled from 3 experiments, $n \geq 8$ cultures per group, $* = p < 0.001$, one-way ANOVA post-hoc Scheffe test). B) Rottlerin application to distal axons prevents apoptosis of compartmented cultures deprived of NGF for 72 h (data compiled from 3 experiments, $n \geq 5$ cultures per group, $* = p < 0.001$, one-way ANOVA post-hoc Scheffe test). C) Rottlerin does not induce apoptosis in cultures supplied with NGF at their distal axons (data compiled from 2 experiments, $n = 5$ cultures per group).


A
*
*
100
80
60
40
20
0
% apoptosis
DAx
CB /PAx
DAx
CB /PAx
DAx
CB /PAx
No Drug
Rott
Chel
Gö6976
B
*
100
80
60
40
20
0
% apoptosis (72 h)
Rott (DAx)
C
100
80
60
40
20
0
% apoptosis
DAx
CB /PAx
No Drug
Rott

cell bodies/proximal axons (6%) or the distal axons (4%) compared to no drug controls (4%).

When chelerythrine was tested in NGF-deprived cultures, the results were similar to those obtained for rottlerin; only application of chelerythrine (10 μM) to the distal axons significantly reduced apoptosis to 48% from the 84% observed for no drug controls (Figure 4-4A). Application of chelerythrine (10 μM) to the cell bodies/proximal axons was not protective (86% apoptosis). Thus, the results of the experiment assaying apoptosis correlated with the experiment examining *c*-jun activation. Rottlerin and chelerythrine application to the distal axons prevented both these apoptotic events.

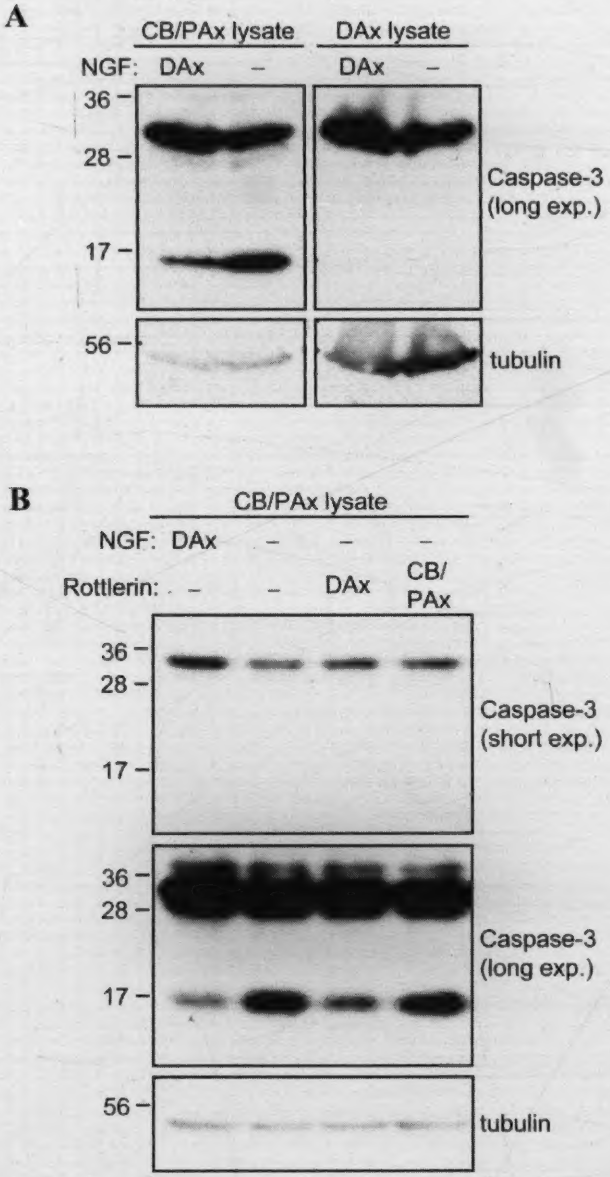
Rottlerin and chelerythrine both inhibit PKCs. Rottlerin is reported to be specific for the novel PKC isoform, PKCδ, whereas chelerythrine is reported to be a general PKC inhibitor, acting on both conventional and novel PKC isoforms. To distinguish if chelerythrine was mediating its effects by acting on conventional or novel PKCs, a third PKC inhibitor, Gö6976, was tested. Gö6976 was used as a TrkA inhibitor in the previous chapter but was originally identified as an inhibitor of conventional PKC isoforms (Gschwendt et al., 1995). As expected from the results in the previous chapter, application of 25 nM Gö6976 to either the cell bodies/proximal axons (83% apoptosis) or the distal axons (80% apoptosis) did not provide significant protection from apoptosis in response to NGF deprivation (Figure 4-4A).

Since the PKC isoform, PKCδ, was a target of inhibition shared by rottlerin and chelerythrine, but not Gö6976, it was initially hypothesized that PKCδ might participate in generating the axon apoptotic signal. However, data presented later in this study (Section 4.3.1) did not support this hypothesis.

4.2.5 Rottlerin prevents cleavage of caspase 3 only when applied to distal axons

It was next determined if blocking the axon apoptotic signal with rottlerin also prevented another major apoptosis signaling event, caspase-3 cleavage. Cleavage of caspase-3 is a late event in sympathetic neuron apoptosis (Deshmukh and Johnson, 1997; 1998). Sympathetic neurons isolated from caspase-$3^{-/-}$ mice were protected from apoptosis induced by NGF deprivation (Wright et al., 2007), which suggests that caspase-3 function is essential for this process. It was first assessed if caspase-3 cleavage occurred in the cell bodies/proximal axons and distal axons during apoptosis by depriving cultures of NGF for 21 h, then analyzing the lysates by immunoblot for caspase-3. A strong signal for full length caspase-3 migrating at approximately 30 kDa (predicted MW = 32 kDa) was observed in both the distal axons and cell bodies/proximal axons in both the control cultures supplied with NGF at distal axons and the NGF-deprived cultures (Figure 4-5A). In contrast, the 17 kDa cleavage product of caspase-3 was only detected in the cell bodies/proximal axons, and the intensity of the signal was increased in NGF-deprived cultures compared to NGF-supplied control cultures. It was next assessed if rottlerin supplied to the distal axons or the cell bodies/proximal axons during NGF deprivation prevented the cleavage of caspase-3 occurring at 21 h. Lysates of the cell bodies/proximal axons from treatment groups were analyzed by immunoblot for caspase-3. Rottlerin (2.5 μM) blocked the increase in the cleaved caspase-3 product (17 kDa) only when supplied to the distal axons during NGF deprivation (Figure 4-5B). Thus, rottlerin applied to the distal axons prevented apoptosis as measured by three different apoptotic indicators: *c-jun* activation, caspase-3 cleavage, and nuclear condensation.

Figure 4-5. Rottlerin supplied to the distal axons prevents activating cleavage of caspase-3 during NGF deprivation. Distal axons (DAx) were supplied with 50 ng/ml NGF where indicated, otherwise NGF antibodies (–) were supplied. Cell bodies/proximal axons were treated with NGF antibodies in all cultures. Rottlerin (2.5 μM) was supplied in the specified compartments during treatment. Lysates were analyzed by immunoblot after 21 h of treatment. The blot was probed with an antibody recognizing the p35, full-length caspase-3 and the p17, cleaved form. The cleaved form was visualized only after long exposure times. The blot was reprobed for β-tubulin as a loading control. Results are representative of 3 independent experiments. A) Caspase-3 cleavage occurs in the cell bodies/proximal axons but not in distal axons at 21 h of NGF deprivation. B) Rottlerin application to distal axons during NGF deprivation decreases caspase-3 cleavage.

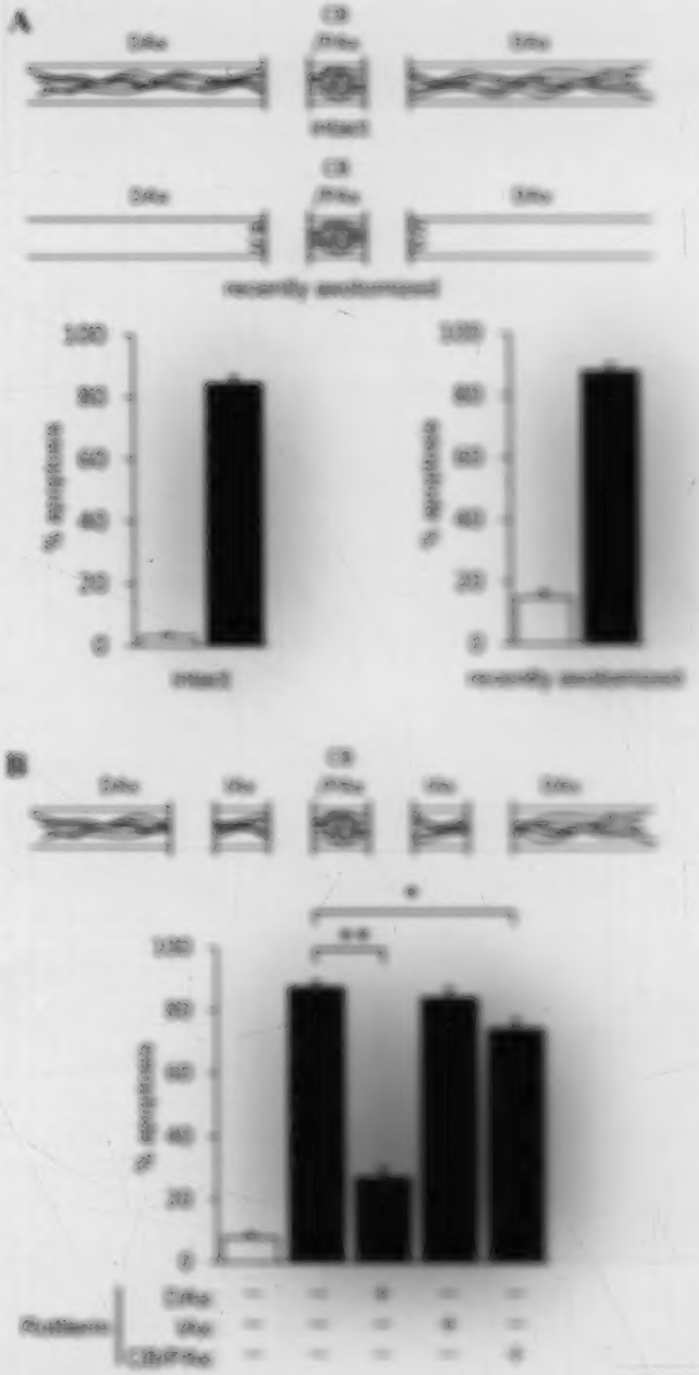

A
CB/PAx lysate
DAx lysate
NGF: DAx –
DAx –
36
28
17
Caspase-3 (long exp.)
56
tubulin
B
CB/PAx lysate
NGF: DAx – – –
Rottlerin: – – DAx CB/PAx
36
28
17
Caspase-3 (short exp.)
36
28
17
Caspase-3 (long exp.)
56
tubulin

4.2.6 Rottlerin blocks apoptosis specifically from axon terminal segments

Since previous experiments provided evidence that an apoptotic signal from axons was required for apoptosis, a plausible hypothesis is that shortening the distal axons would decrease this signal enough to prevent apoptosis. The axons in the distal axon compartment were removed (termed axotomy) by a combined method of mechanical disruption and osmotic lysis. The cultures were allowed to recover for 6 h in medium containing 50 ng/ml NGF during which time, axons began to re-extend a short distance into the distal compartment. Based on previously reported rates of axon extension of 1.3 mm/day (MacInnis et al., 2003), the axons were estimated have extended about 325 μm. These cultures with short distal axons were then completely deprived of NGF for 24 h and assessed for apoptosis. Whereas the majority of neurons supplied with NGF at their distal axons survived (15% apoptosis), cultures deprived of NGF underwent 88% apoptosis (Figure 4-6A). This level of apoptosis was similar to that observed in intact cultures with long distal axons (84% apoptosis), suggesting that even short distal axons are capable of generating an effective axon apoptotic signal. This is additionally supported by data in mass cultures that neurons deprived of NGF after only 2 days in cultures (relatively short axons) still underwent apoptosis in 24 h (Figure 4-10).

An explanation that may account for the above results is that the axon apoptotic signal can be effectively generated from a small axon segment, perhaps from a specialized region of the axon such as the axon terminals. To test this possibility, sympathetic neurons were grown in 5-compartment cultures. In these cultures, neurons extended their axons across one barrier into the 1 mm wide intermediate axon compartment, then across a second barrier into the distal axons compartment (Figure 4-

Figure 4-6. Rottlerin blocks the axonal apoptotic signal at axon terminals. A) Compartmented cultures with shortened distal axons undergo apoptosis similarly to cultures with intact distal axons. The distal axons (DAx) of compartmented cultures were removed by axotomy (see Section 2.7 for details) and allowed to regrow for 6 h in medium containing 50 ng/ml NGF. The regrown distal axons were then switched to medium containing NGF antibodies (black bar) or resupplied with medium containing 50 ng/ml NGF (white bar) for 24 h. Cell bodies/proximal axons of both groups were given medium containing NGF antibodies. Apoptosis was identified by condensed, fragmented nuclei at 24 h. The mean percentage of apoptotic neurons per group from data compiled from 3 experiments is plotted (± SEM, n = 9 cultures). The same NGF deprivation experiment carried out in non-axotomized (intact) cultures is also graphed for comparison (data compiled from 3 experiments, ± SEM, n = 9 cultures). B) Sympathetic neurons were cultured in five-compartmented cultures. After 5 days in culture, NGF was removed from intermediate axons (IAx) and cell bodies/proximal axons (CB/PAx) compartments for two days to induce axon terminals in these compartments to degenerate and to apoptose neurons whose axons had not yet crossed into the compartments containing the distal axons (DAx). Following this pretreatment, cell bodies/proximal axons and intermediate axons were given medium containing NGF antibodies and distal axons were treated with 50 ng/ml NGF (white bar), NGF antibodies (black bars), and rottlerin (Rott, 2.5μM) where indicated for 24 h. Apoptosis was assessed as in A). Data plotted for each group is compiled from three experiments (± SEM, $n \geq 7$ cultures, * = $p < 0.05$, ** = $p < 0.001$, one-way ANOVA post-hoc Scheffe test).

A
intact
recently axotomized
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0
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0
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80
100

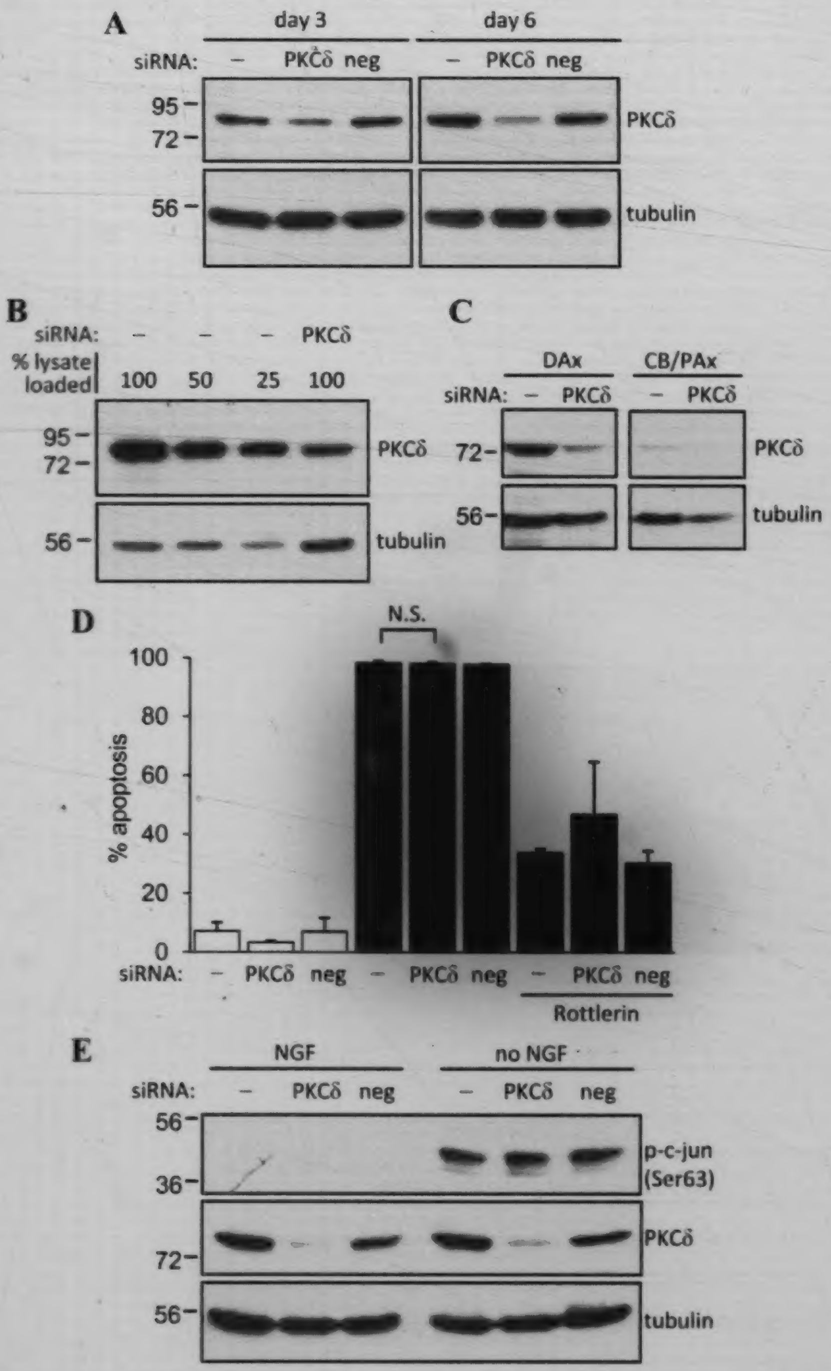
6B). After 5 days in culture, the intermediate axons and the cell bodies/proximal axons were pre-treated with medium lacking NGF for 2 days during which the distal compartments provided the only source of NGF. Neurons with axons that had not yet crossed into the distal axons compartment and failed to receive NGF would be eliminated by apoptosis (Campenot, 1982b). In addition, axon branches that terminated in the compartments containing the intermediate axons and cell bodies/proximal axons would degenerate (Campenot, 1982a). This pretreatment resulted in compartment cultures selected for neurons with axons extending into and terminating within the distal axon compartments. At this point, the entire culture was deprived of NGF for 24 h in the presence of rottlerin (2.5 μM) supplied to various compartments as indicated in Figure 4-6B, and then assessed for apoptosis. NGF deprivation resulted in the apoptosis of 87% of neurons. In cultures supplied with rottlerin on their distal axons, this rate was substantially reduced to only 27% (Figure 4-6B). Application of rottlerin to the intermediate axons did not provide significant protection (84% apoptosis). Rottlerin applied to the cell bodies/proximal axons provided a modest but significant amount of protection, reducing apoptosis to 74%. Since the majority of rottlerin's protective effect was observed when the drug was applied to the distal axons containing axon terminal segments, the results suggest that rottlerin blocks an axon apoptotic signaling event localized to axon terminals. These terminals may serve as initiation sites for the axon apoptotic signal. *In vivo*, target-derived NGF acts on axon terminals. Therefore, a major mechanism by which NGF signaling at axon terminals promotes survival may be by locally suppressing the generation of the axon apoptotic signal.

4.3 Results-Characterizing signaling components of the axon apoptotic signal during NGF deprivation

4.3.1 Knockdown of PKCδ by siRNA is not protective against apoptosis

Rottlerin and chelerythrine both share a common target: PKCδ. Therefore, it is hypothesized that PKCδ is a component of the axon apoptotic signal. PKCδ is implicated in promoting apoptosis in models of glutamate toxicity in primary cortical neurons (Choi et al., 2006) and neurotoxin responses in dopaminergic neurons (Kaul et al., 2003), which provide a precedent for a pro-apoptotic function for PKCδ. The protein level of PKCδ in sympathetic neurons was reduced by transfecting them with a pool of 4 siRNAs targeting rat PKCδ mRNA. Using a pool of siRNAs reduced the concentration of each siRNA thereby decreasing the probability of silencing non-specific targets. Transfection was carried out on dissociated sympathetic neurons immediately prior to their plating in mass cultures by a technique termed nucleofection (See Section 2.6.1). Two types of control transfections were also performed:1) transfection without siRNA (−) and 2) transfection with a negative control siRNA (neg) that should not target any known rat mRNAs. Immunoblot analysis of lysates from transfected neurons at 3 and 6 days in culture showed a reduction of PKCδ protein levels compared to the no siRNA and negative control siRNA groups (Figure 4-7A). Comparison of the signal from the PKCδ-transfected sample to dilution fractions of the no siRNA control sample showed that PKCδ protein had been decreased to less than 25% of the control (Figure 4-7B). To determine if loss of PKCδ was occurring in axons, transfected neurons were plated in compartmented cultures. Immunoblots of cultures 3 days post-transfection showed decreases in PKCδ in both the cell bodies/proximal axons and distal axons (Figure 4-7C).

Figure 4-7. Knockdown of PKCδ by siRNA does not prevent NGF deprivation-induced apoptosis. Sympathetic neurons were transfected by nucleofection (see Materials and Methods for details) with a pool of 4 siRNAs (PKCδ) targeting rat PKCδ mRNA prior to plating in mass culture. Transfections were also carried out with no siRNA (–) or a non-specific siRNA (neg) as controls. A) PKCδ knockdown at the protein level. Transfected neurons were harvested on day 3 or 6 in culture as indicated, and equivalent amounts of cell lysates (8 μg) were analyzed by immunoblot. The blot was probed with an antibody against PKCδ and reprobed for β-tubulin as a loading control. Results are representative of 3 independent experiments. B) Quantification of PKCδ knockdown. Day 4 cultures were harvested and loaded onto an SDS-PAGE gel in the relative amounts indicated (8 μg = 100%). The blot was probed for PKCδ and reprobed for β-tubulin as a loading control. Results are representative of 2 independent experiments. C) Knockdown of PKCδ occurs in distal axons. Transfected neurons were plated in compartmented cultures and analyzed by immunoblot after 3 days. The blot was probed for PKCδ and reprobed for β-tubulin as a loading control. Results are representative of 2 independent experiments. D) Apoptosis in response to NGF withdrawal. Day 5 transfected neurons in mass culture were maintained in NGF (white bars) or deprived of NGF (black bars) for 24 h. Rottlerin (2.5 μM) was supplied where indicated. Apoptosis was identified by condensed, fragmented nuclei at 24 h. The percentage of apoptotic neurons in each group is plotted (± SEM, n = 4 cultures, N.S. = not significant, one-way ANOVA post-hoc Tukey HSD). E) *c*-jun activation during NGF deprivation. Transfected neurons cultured for 3 days were maintained in NGF (NGF) or deprived of NGF (no NGF) for 6 h, then harvested and analyzed by immunoblot. Blots were probed with an antibody recognizing phosphorylated *c*-jun (Ser63). Blots were reprobed for PKCδ and β-tubulin. Results are representative of 3 independent experiments.


A
day 3
day 6
siRNA: – PKCδ neg – PKCδ neg
95
72
56
PKCδ
tubulin
B
siRNA: – – – PKCδ
% lysate loaded 100 50 25 100
95
72
56
PKCδ
tubulin
C
DAx
CB/PAx
siRNA: – PKCδ – PKCδ
72
56
PKCδ
tubulin
D
N.S.
% apoptosis
100
80
60
40
20
0
siRNA: – PKCδ neg – PKCδ neg – PKCδ neg
Rottlerin
E
NGF
no NGF
siRNA: – PKCδ neg – PKCδ neg
56
36
72
56
p-c-jun (Ser63)
PKCδ
tubulin

NGF deprivation resulted in apoptotic rates that did not differ between neurons transfected with PKCδ siRNA (98%), no siRNA (98%), or the negative control siRNA (98%) suggesting that reduction of PKCδ protein was not protective (Figure 4-7D). Rottlerin was able to rescue neurons from apoptosis in all transfected groups. Knockdown of PKCδ was also unable to prevent the phosphorylation of *c*-jun induced by NGF deprivation (Figure 4-7E). Although it cannot be ruled out that residual PKCδ (less than 25% of controls) was sufficient for generating the axon apoptotic signal in these experiments, the data obtained does not support a role for PKCδ in the axon apoptotic signal,

4.3.2 Rottlerin applied to distal axons inhibits the activation of GSK3

Since there was no evidence to support a role for PKCδ in promoting apoptosis, it suggests that rottlerin produces its anti-apoptotic effects by acting on another molecule. One reported target of rottlerin identified by *in vitro* kinase assays is glycogen synthase kinase-3 beta (GSK3β). GSK3β can function as an anti-apoptotic or pro-apoptotic kinase depending on the cell type or stimulus (Beurel and Jope, 2006). The activation of GSK3 during NGF deprivation was assessed by monitoring the phosphorylation of the closely related isoforms, GSK3α and GSK3β (Woodgett, 1990). GSK3s are activated by dephosphorylation at Ser21 of GSK3α and Ser9 of GSK3β (Sutherland and Cohen, 1994; Sutherland et al., 1993). Immunoblot analysis revealed that in cultures deprived of NGF for 6 h, both GSK3 isoforms were dephosphorylated (activated) in the distal axons compared to control cultures supplied with NGF (Figure 4-8, lanes 5 and 6). Loss of phosphorylation was not due to loss of total GSK3 levels since they remained unchanged. Application of 2.5 μM rottlerin to the distal axons during NGF deprivation partially

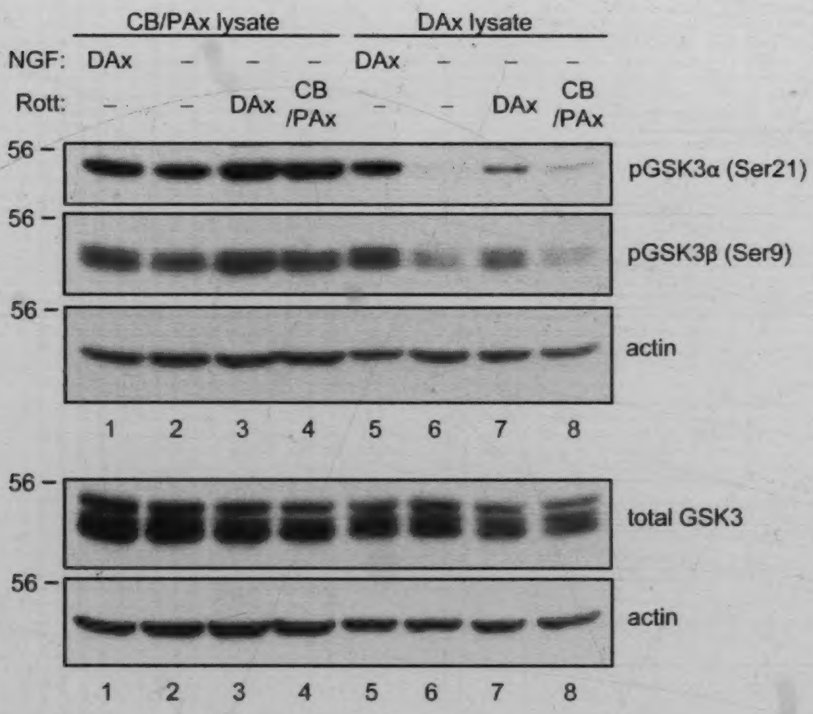

CB/PAx lysate
DAx lysate
NGF: DAx – – – DAx – – –
Rott: – – DAx CB /PAx – – DAx CB /PAx
56 –
pGSK3α (Ser21)
pGSK3β (Ser9)
actin
1 2 3 4 5 6 7 8
total GSK3
actin
1 2 3 4 5 6 7 8

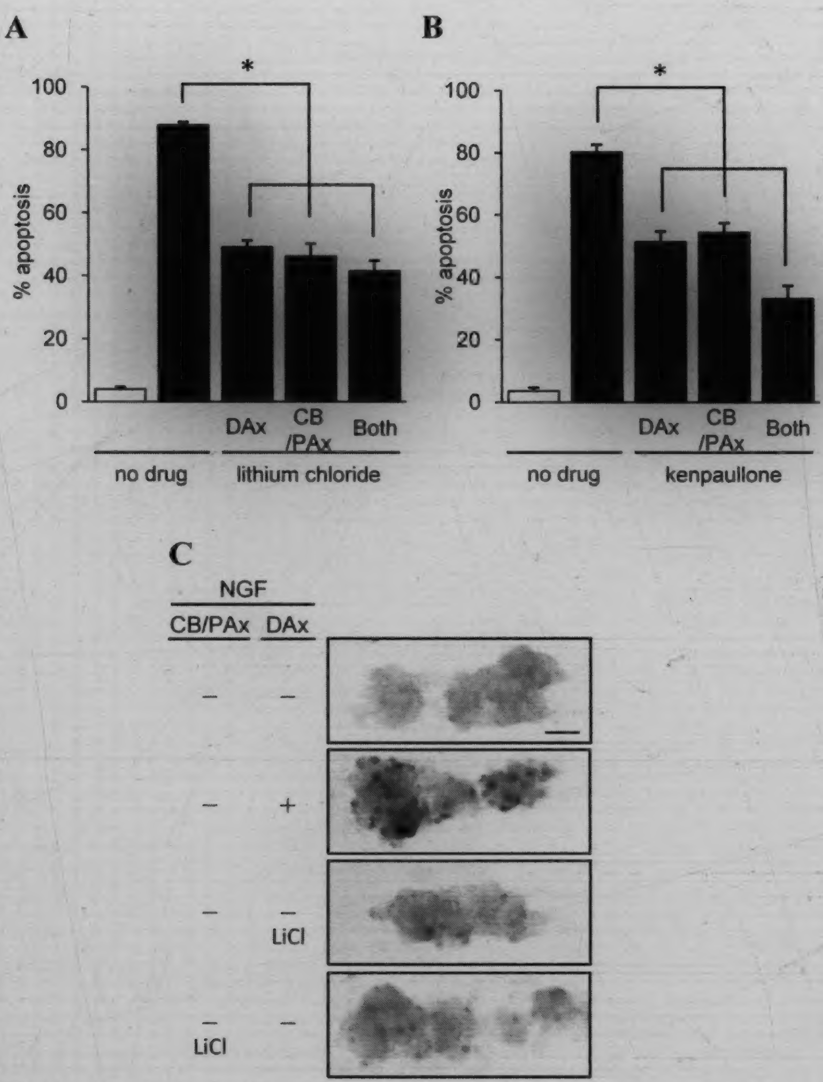
Figure 4-8. Rottlerin applied to the distal axons inhibits the activation of GSK3 that occurs during NGF deprivation. Rottlerin inhibits activation (dephosphorylation) of GSK3α and GSK3β. Distal axons (DAx) were treated with 50 ng/ml NGF or with NGF antibodies (-). Cell bodies/proximal axons (CB/PAx) were treated with NGF antibodies in all cultures. Rottlerin (2.5 μM) was supplied to compartments for the duration of treatment as indicated. Lysates from each group were split into two equal samples and analyzed on separate immunoblots following 6 h of treatment. One blot was probed with an antibody recognizing phosphorylated GSK3α (Ser21) and GSK3β (Ser9), then probed for actin as a loading control. The other blot was probed for total GSK3 (both alpha and beta isoforms), then probed for actin as a loading control. Results are representative of 3 independent experiments.

blocked the dephosphorylation of GSK3. The finding that rottlerin prevented the activation of GSK3 suggests that rottlerin does not inhibit GSK3 directly, but rather acts on the pathway lying upstream of GSK3 activation. Application of 2.5 μM rottlerin to the cell bodies/proximal axons did not block GSK3 dephosphorylation in the distal axons (Figure 4-8, lanes 7 and 8). In examining the cell bodies/proximal axons lysates, there also appeared to be a slight dephosphorylation of GSK3 in response to NGF deprivation (Figure 4-8, lanes 1 and 2) that was inhibited by rottlerin application to the distal axons (lane 3) but not to the cell bodies/proximal axons (lane 4). Although the differences were small, it suggests that the slight activation of GSK3 in the cell bodies/proximal axons may be linked to the axon apoptotic signal inhibited by rottlerin. The finding that rottlerin applied to distal axons prevents GSK3 activation raised the possibility that GSK3 functions in the axon apoptotic signaling pathway.

4.3.3 GSK3 inhibitors and GSK3 siRNA knockdown provide evidence that GSK3 promotes apoptosis of sympathetic neurons

It was next determined if GSK3 activity is required for the apoptosis induced by NGF deprivation. Two inhibitors of GSK3, lithium chloride (LiCl) and kenpaullone (Bain et al., 2007) were tested for their effects on apoptosis. These inhibitors block activity of both GSK3α and GSK3β (Klein and Melton, 1996; Leost et al., 2000; Phiel et al., 2003). Compartmented cultures were deprived of NGF for 24 h during which time 15 mM LiCl or 5 μM kenpaullone were provided to compartments as indicated in Figure 4-9. NGF-deprived neurons underwent 88% apoptosis and LiCl supplied to the distal axons reduced this rate to 49% (Figure 4-9A). LiCl application to the cell bodies/proximal axons also reduced apoptosis (46%). LiCl applied to the entire neuron resulted in 41%

Figure 4-9. GSK3 inhibitors, LiCl and kenpaullone, prevent apoptosis induced by NGF deprivation. A and B) GSK3 inhibitors reduce apoptosis during NGF deprivation. Cell bodies/proximal axons (CB/PAx) of compartmented cultures were supplied with NGF antibodies and distal axons (DAx) were supplied with either 50 ng/ml NGF (white bars) or NGF antibodies (black bars). Lithium chloride (15 mM) or kenpaullone (5 μM) was provided to the compartments indicated during treatment. Apoptosis was identified by condensed, fragmented nuclei and plotted as percentage of apoptotic neurons per group (± SEM, n = 9 cultures, * = $p<0.001$, one-way ANOVA post-hoc Scheffe test). C) LiCl application to the distal axons or cell bodies/proximal axons prevents *c*-jun activation. Distal axons (DAx) were supplied with 50 ng/ml NGF (+) or medium lacking NGF and containing NGF antibodies (–) as indicated. All cell bodies/proximal axons (CB/PAx) were treated with NGF antibodies (–). LiCl was supplied to the compartments indicated for the duration of treatments. The cultures were processed for detection of phosphorylated *c*-jun at 6 h of treatment as in Figure 4-1A. The scale bar equals 50 μm.


A
*
100
80
60
40
20
0
% apoptosis
DAx
CB /PAx
Both
no drug
lithium chloride
B
*
100
80
60
40
20
0
% apoptosis
DAx
CB /PAx
Both
no drug
kenpaullone
C
NGF
CB/PAx
DAx
–
–
–
+
–
–
LiCl
–
LiCl
–

apoptosis, which was similar to the rates observed for LiCl supplied to either compartment indicating the protective effects were not additive. Similar protective effects were observed with kenpaullone (Figure 4-9B). NGF deprivation resulted in 80% apoptosis, and supplying kenpaullone to the distal axons or the cell bodies/proximal axons partially prevented apoptosis (51% and 54% apoptosis, respectively). Interestingly, kenpaullone application to both compartments further reduced apoptosis to 33%, suggesting that kenpaullone's protective effects within the different compartments were to some degree additive.

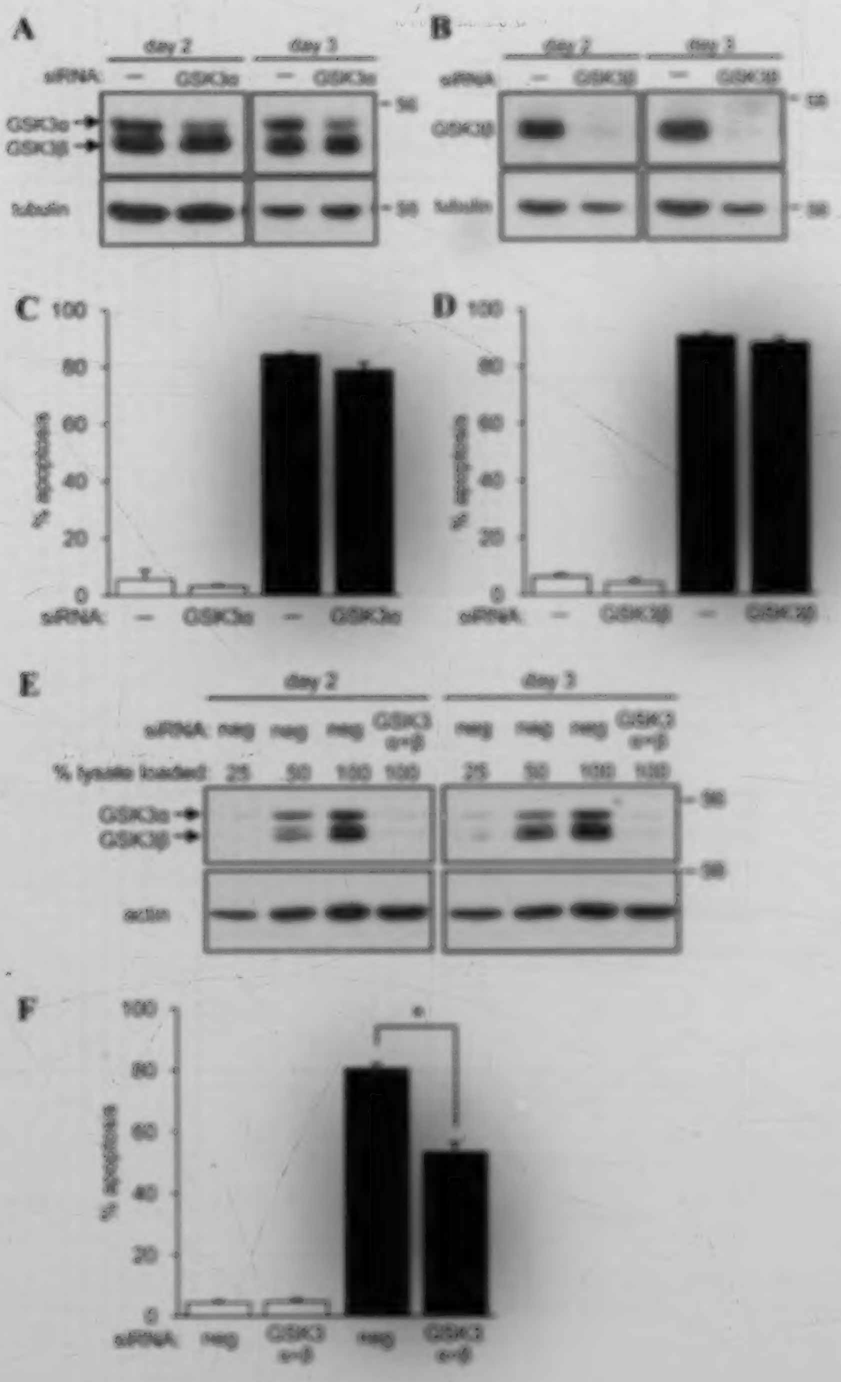
The results with the GSK3 inhibitors support the hypothesis that rottlerin acts upstream of GSK3 activation, since rottlerin was only protective when applied to the distal axons but direct inhibition of GSK3 was protective when applied to the distal axons, as well as, the cell bodies/proximal axons. The finding that GSK3 inhibitors are protective when applied to either compartment suggests that GSK3 activity is required in both the distal axons and the cell bodies to promote apoptosis. The results are also consistent with the possibility that GSK3 plays a role in carrying the apoptotic signal from the distal axons to the cell bodies, since inhibiting GSK3 in either compartment would block the transmission of the signal.

Immunoblot analysis in the previous Section showed that only a small portion of GSK3 in the cell bodies/proximal axons was activated in response to NGF deprivation (Figure 4-8). The data obtained with the GSK3 inhibitors suggest that the small amount of activated GSK3 was sufficient for mediating downstream apoptotic signaling. If GSK3 does play a role in carrying the retrograde apoptotic signal, then the small amount of activated GSK3 observed in the cell bodies/proximal axons may be a result of transport of activated GSK3 from the distal axons.

To verify GSK3's involvement in the axon apoptotic signal, the effects of GSK3 inhibition on the activation of *c*-jun was assessed. Compartmented cultures were deprived of NGF for 6 h in the presence of 15 mM LiCl supplied to the distal axons or to the cell bodies/proximal axons. The cell bodies/proximal axons were then immunohistochemically stained for phosphorylated *c*-jun. Supplying LiCl to either the distal axons or the cell bodies/proximal axons during NGF deprivation largely prevented the appearance of nuclear accumulated phosphorylated *c*-jun (Figure 4-9C). This suggests that GSK3 activity in the distal axons and the cell bodies/proximal axons is required by the axon apoptotic signal to induce *c*-jun activation.

To support the evidence obtained with the GSK3 inhibitors suggesting that GSK3 activity promoted apoptosis, GSK3 was knocked down using pools of siRNAs against GSK3α and/or GSK3β. For GSK3β, the pool was composed of 4 different siRNAs. For GSK3α, 4 siRNAs were tested for their ability to reduced GSK3α protein levels and only the 2 most effective were used in the pool. Transfection with either GSK3α or GSK3β siRNA resulted in the reduction of the corresponding proteins compared to control cultures transfected without siRNA (–), as confirmed by immunoblot analysis on day 2 and day 3 post-transfection (Figures 4-10A and 4-10B). However, reduction of either GSK3α or GSK3β protein did not significantly prevent the apoptosis of transfected mass cultures in response to NGF deprivation initiated 2 days post-transfection (Figure 4-10C and 4-10D). One possible reason for the results obtained is that GSK3α and GSK3β are functionally redundant with respect to apoptotic activity. Therefore, experiments were

Figure 4-10. Double siRNA knockdown of GSK3α and GSK3β decreases apoptosis in response to NGF withdrawal. Sympathetic neurons were transfected by nucleofection with siRNAs targeting rat GSK3α mRNA (GSK3α), rat GSK3β mRNA (GSK3β), or both mRNAs (GSK3α+β) prior to plating in mass culture. Control transfections were also carried out with no siRNA (–) or non-targeting siRNA (neg). A, B, and E) Quantification of knockdown. Day 2 and day 3 cultures were harvested and loaded onto SDS-PAGE gels in the relative amounts indicated (10 μg = 100%). The immunoblot was probed with an antibody recognizing total GSK3α and GSK3β or only GSK3β (GSK3β knockdown experiment). The blot was reprobed for α-tubulin or actin as a loading control. Results are representative of 3 independent experiments. A) GSK3α knockdown. B) GSK3β knockdown experiment. E) GSK3α+β knockdown. C, D, and F) Apoptosis in response to NGF deprivation. Transfected neurons cultured in mass for 2 days were maintained in NGF (white bars) or deprived NGF (black bars) for 24 h. Apoptosis was identified by condensed, fragmented nuclei and plotted as percentage of apoptotic neurons per group (±SEM). C) Apoptosis result for GSK3α knockdown. D) Apoptosis result for GSK3β knockdown. F) Apoptosis result for GSK3α+β knockdown (n = 9 cultures, compiled from 3 experiments, * = $p < 0.001$, one-way ANOVA post-hoc Tukey HSD).


A
day 2
day 3
siRNA: — GSK3α — GSK3α
GSK3α
GSK3β
tubulin
50
50
B
day 2
day 3
siRNA: — GSK3β — GSK3β
GSK3β
tubulin
50
50
C
100
80
60
40
20
0
% apoptosis
siRNA: — GSK3α — GSK3α
D
100
80
60
40
20
0
% apoptosis
siRNA: — GSK3β — GSK3β
E
day 2
day 3
siRNA: neg neg neg GSK3 α+β neg neg neg GSK3 α+β
% lysate loaded: 25 50 100 100 25 50 100 100
GSK3α
GSK3β
actin
50
50
F
100
80
60
40
20
0
% apoptosis
*
siRNA: neg GSK3 α+β neg GSK3 α+β

carried out in which both GSK3 isoforms were knocked down. Transfection with siRNA against both GSK3α and GSK3β resulted in a 75% decrease in protein levels of both GSK3 isoforms in comparison to the negative control siRNA transfected cultures, as determined by immunoblot of mass culture lysates at day 2 and day 3 (Figure 6-10E). When levels of both GSK3 isoforms were reduced, apoptosis was reduced to 33% compared to the 80% observed in control neurons transfected with a negative control siRNA (Figure 6-10F).

In summary, the results of the GSK3 inhibitor and GSK3 siRNA experiments provide evidence that GSK3 promotes the apoptosis of sympathetic neurons in response to NGF deprivation. The results obtained with the GSK3 inhibitors suggest that GSK3 promotes the retrograde apoptotic signal at both the distal axons and cell bodies/proximal axons, possibly by functioning as a carrier for the signal.

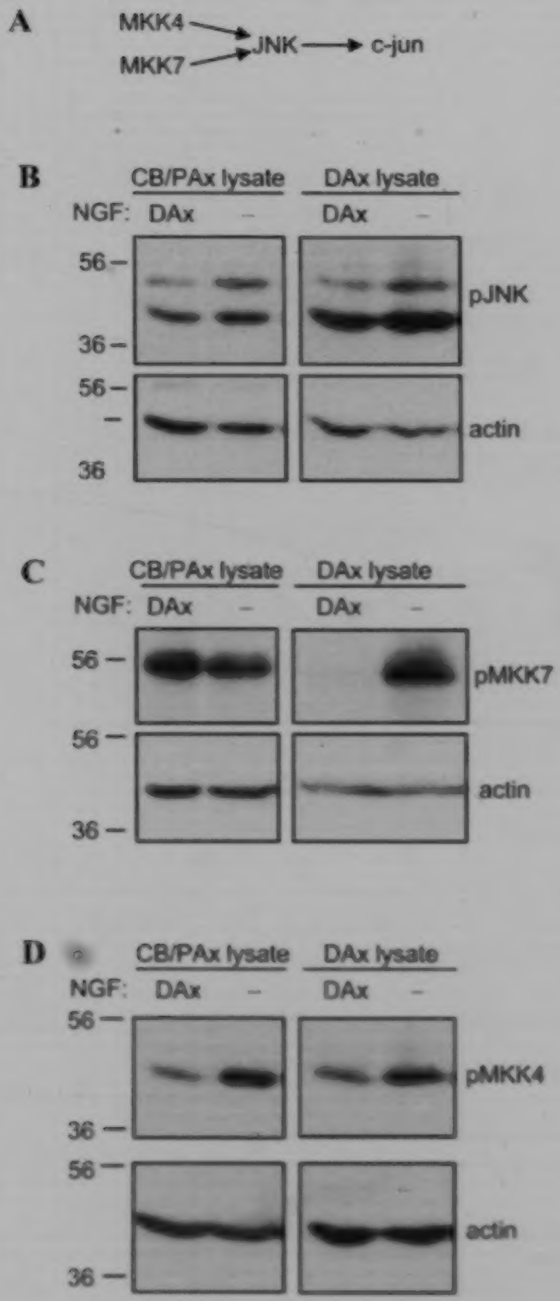
6.3.4 Signaling molecules known to function upstream of *c-jun* are activated in the distal axons during NGF deprivation

The results presented in this study place the axon apoptotic signal upstream of c-jun activation. Several of the signaling events that occur upstream of c-jun activation in NGF deprived sympathetic neurons have been previously characterized. c-jun NH2-terminal kinases (JNKs) directly phosphorylate c-jun at Ser^{63} and Ser^{73} (Derijard et al., 1994; Hibi et al., 1993). There are 3 JNK genes that give rise to 12 identified isoforms in total (Kyriakis and Avruch, 2001). JNKs are activated via phosphorylation by the kinases MKK4 (Sanchez et al., 1994) and MKK7 (Tournier et al., 1997). Although the activation of these kinases in sympathetic neurons during NGF deprivation has been documented previously (Eilers et al., 2001; Harding et al., 2001; Xu et al., 2001), activation was not specifically demonstrated in axons. Compartmented culture neurons were deprived of NGF for 6 h, and the phosphorylation state of JNKs, MKK4, and MKK7 was analyzed. For the immunoblot of phosphorylated JNKs, an antibody recognizing activating phosphorylation of Thr183 and Tyr185 of all JNK isoforms was used. Cultures maintained with NGF on the distal axons revealed basal phosphorylation of JNKs in both the distal axons and cell bodies/proximal axons (Figure 4-11B). NGF deprivation resulted in increased JNK phosphorylation in both the distal axons and cell bodies/proximal axons. MKK4 and MKK7 activation was assayed with antibodies recognizing MKK4 phosphorylated at Ser257 and Thr261 and MKK7 phosphorylated at Ser271 and Thr275 (Tibbles et al., 1996; Wang et al., 2007). Increased phosphorylation of both MKK4 and MKK7 in the distal axons was observed in response to NGF deprivation (Figure 4-11C and 4-11D). Increased phosphorylation in the cell bodies/ proximal axons was also detected for MKK4 but not MKK7, which had a high basal level of phosphorylation. Our results indicate that signaling components upstream of *c-jun* are activated in the distal axons in response to NGF deprivation and are, therefore, possibly components of the axon apoptotic signaling pathway. The observation that some of these molecules are also activated in the cell bodies/proximal axons may signify that these activated kinases function as retrograde carriers of the axon apoptotic signal.

4.3.5 Rac1 may function in two apoptotic pathways: the axon apoptotic signal and another mechanism located in the cell bodies/proximal axons

Rac1 is a GTPase that is implicated in sympathetic neuron apoptosis (Bazenet et el., 1998). Rac1 was reported to function upstream of *c-jun* activation, identifying it as a potential component of the axon apoptotic signaling pathway. To begin investigating if

Figure 4-11. Signaling events upstream of *c*-jun activation show increased phosphorylation in response to NGF deprivation. A) The order of the signaling events directly upstream of *c*-jun. Each event consists of activating phosphorylation of a kinase by the kinase directly upstream of it. B-D) Cell bodies/proximal axons (CB/PAx) of compartmented cultures received NGF antibodies while distal axons (DAx) received either 50 ng/ml NGF or NGF antibodies (–). Lysates were collected after 6 h of treatment and processed for immunoblotting. Results are representative of 3 independent experiments. B) Activation of JNK. Immunoblot was probed with an antibody recognizing JNK phosphorylated at Thr 183 and Tyr 185 (pJNK). The blot was reprobed for actin as a loading control. C) Activation of MKK7. Immunoblot was probed with an antibody recognizing MKK7 phosphorylated at Ser 271 and Thr 275 (pMKK7). The blot was reprobed for actin as a loading control. D) Activation of MKK4. Immunoblot was probed with an antibody recognizing MKK4 phosphorylated at Ser 257 and Thr 261 (pMKK4). The blot was reprobed for actin as a loading control.


A
MKK4
MKK7
JNK
c-jun
B
CB/PAx lysate
DAx lysate
NGF: DAx –
DAx –
56 –
36 –
pJNK
56 –
–
36
actin
C
CB/PAx lysate
DAx lysate
NGF: DAx –
DAx –
56 –
pMKK7
56 –
36 –
actin
D
CB/PAx lysate
DAx lysate
NGF: DAx –
DAx –
56 –
36 –
pMKK4
56 –
36 –
actin

Rac1 functions in the axon apoptotic signal, the Rac inhibitor, NSC23766, was tested. NSC23766 blocks Rac activity by preventing the binding of the guanine nucleotide exchange factors Trio and Tiam (Gao et al., 2004). Neurons were deprived of NGF in the presence of 100 μM NSC23766 on the distal axons or the cell bodies/proximal axons for 24 h. As shown in Figure 4-12A, the apoptosis observed during NGF deprivation (84%) was reduced when NSC23766 was applied to either the distal axons (58%) or to the cell bodies/proximal axons (60%). Application of NSC23766 to both compartments reduced apoptosis to 38 %, suggesting that the protective effects of the distal axons and cell bodies/proximal axons were additive.

The Rac inhibitor, NSC23766, was then assayed for its ability to prevent *c*-jun phosphorylation, an event mediated by the axon apoptotic signal. Compartmented culture neurons were deprived of NGF for 6 h with different distributions of 100 μM NSC23766 and then the cellbodies/proximal axon lysates were examined by immunoblot for phosphorylated *c*-jun (Ser63). NGF deprivation induced *c*-jun phosphorylation in the cell bodies/proximal axons and supplying NSC23766 to the distal axons during treatment prevented this phosphorylation (Figure 4-12B). In contrast, application of NSC23766 to the cell bodies/proximal axons did not prevent *c*-jun phosphorylation in the cell bodies/proximal axons. These results are a preliminary indication that Rac1 may function in distal axons but not the cell bodies/proximal axons to promote the axon apoptotic signal that induces *c*-jun activation. Since the Rac inhibitor, NSC23766, prevented apoptosis when supplied to the cell bodies/proximal axons (Figure 4-12A), it must be through a mechanism that is either downstream of *c*-jun activation or in an independent pathway. The finding that NSC23766 supplied to both the distal axons and the cell

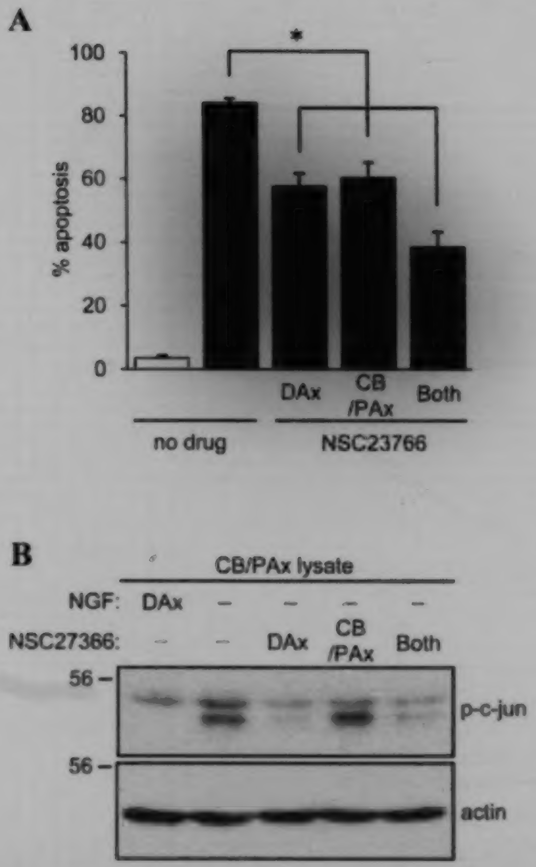


Figure 4-12. The Rac inhibitor, NSC23766, has anti-apoptotic effects when applied to either the distal axons or cell bodies/proximal axons. A) NSC23766 application prevents apoptosis. Cell bodies/proximal axons (CB/PAx) of compartmented cultures received NGF antibodies while distal axons (DAx) were treated with either 50 ng/ml NGF (white bars) or NGF antibodies (black bars). NSC23766 (100 μM) was provided to the distal axons, cell bodies/proximal axons or both compartments during the treatments. Apoptosis was identified at 24 h by condensed, fragmented nuclei and plotted as percentage of apoptotic neurons per group (± SEM, n = 9 cultures, * = $p < 0.001$, one-way ANOVA post-hoc Tukey HSD, data compiled from 3 experiments). B) NSC23766 prevents *c*-jun activation if applied to the distal axons but not the cell bodies/proximal axons. Distal axons (DAx) were supplied with 50 ng/ml NGF or NGF antibodies (–) in the absence or presence of 100 μM NSC23766 where indicated. Cell bodies/proximal axons in all cultures were treated with NGF antibodies. Following a 6 h treatment, cell bodies/proximal axon lysates were analyzed by immunoblotting with an antibody recognizing phosphorylated *c*-jun (Ser63). The blot was reprobed for actin as a loading control. Results are representative of 2 independent experiments.

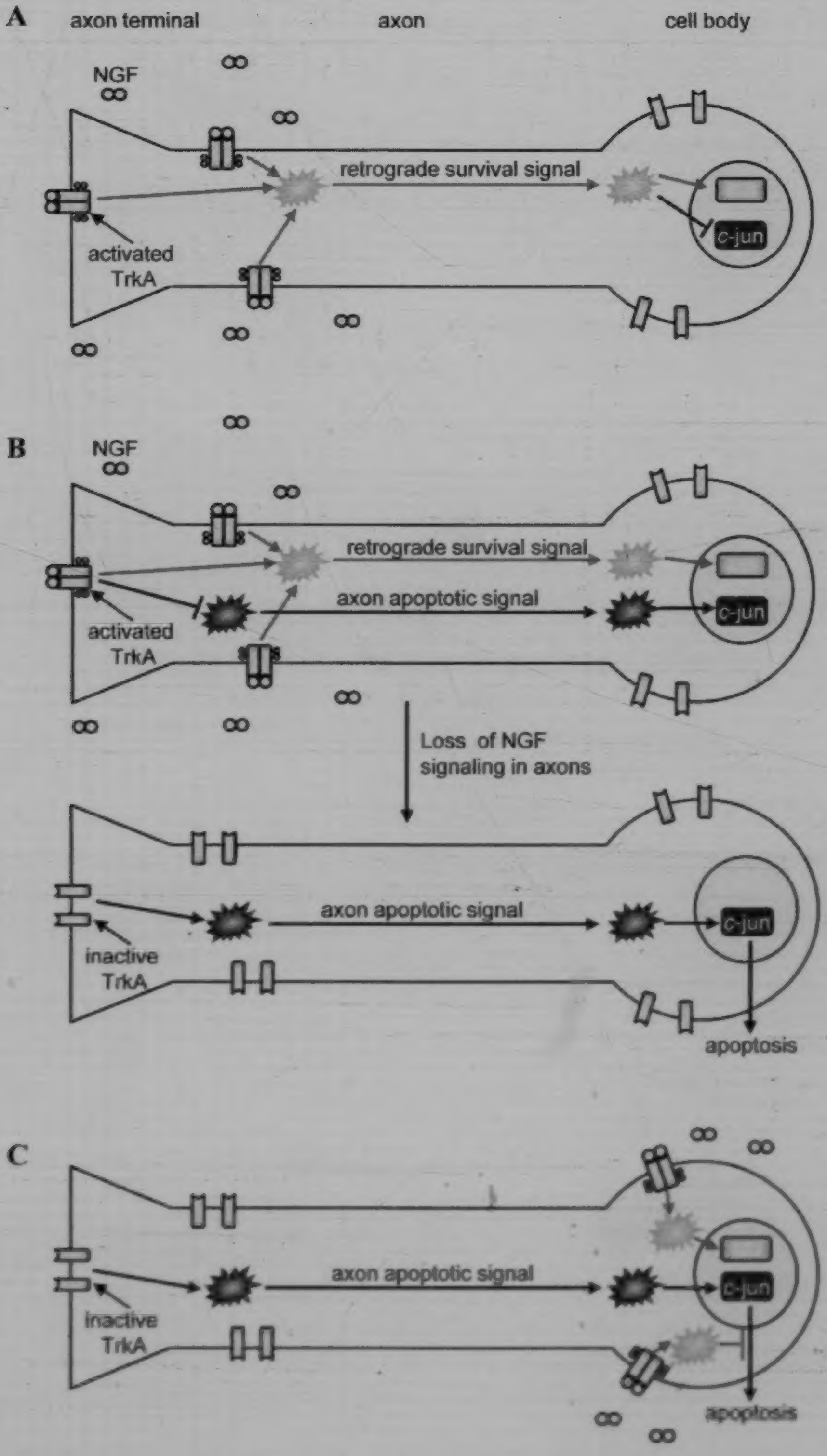
bodies/proximal axons produced an additive protective effect supports the latter mechanism.

4.4 Discussion

The data presented in this study reveal a mechanism for NGF-mediated retrograde survival that is fundamentally different from all previously proposed mechanisms. In previous proposed mechanisms, NGF promotes survival from the axon terminals by generating survival signals that are retrogradely transported to the cell bodies. In contrast, the results of this study demonstrate that NGF also supports survival by suppressing the generation of an axon apoptotic signal that, when induced by withdrawal of NGF from the axons, is transmitted retrogradely to the cell bodies to promote apoptosis.

Are there any alternative explanations for the results of this study other than the existence of an apoptotic signal? One of the main findings of this study is that NGF withdrawal from the distal axons induces that activation of *c*-jun in the cell bodies and that supplying NGF directly to the cell bodies/proximal axons could not block this activation (shown schematically in Figure 4-13). These results were interpreted as evidence that *c*-jun activation was not caused by absence of survival signaling at the cell bodies/proximal axons but rather the absence of NGF signaling at the distal axons which resulted in the generation of a retrograde apoptotic signal. An alternative explanation that could be offered is that NGF signaling in the distal axons but not the cell bodies/proximal axons generates a unique survival signal that prevents *c*-jun activation. However, the observation that blocking microtubule-based transport with colchicine prevents the phosphorylation of *c*-jun in the cell bodies/proximal axons induced by NGF deprivation

Figure 4-13.Fitting the axon apoptotic signal in the context of previous NGF retrograde signaling mechanisms. A) Previously proposed mechanisms for how NGF-TrkA signaling initiated in axons promotes survival involve the generation of retrograde survival signals (green). These retrograde survival signals are transmitted to the cell bodies to promote survival signaling and gene expression and to suppress apoptotic signaling (blue) such as activation of the transcription factor *c*-jun. B) The discovery of the axon apoptotic signal adds a new dimension to NGF retrograde signaling. NGF signaling via TrkA in axons generates retrograde survival signals (green) and suppresses the generation of the axon apoptotic signal (blue) from axon terminals. When the axons are deprived of NGF, the axon apoptotic signal is generated and is transmitted back to the cell bodies to activate *c*-jun. C) The axon apoptotic signal is not deactivated in the cell bodies by competing NGF-initiated survival signals. NGF added directly to the cell bodies/proximal axons of neurons in compartmented cultures generates survival signals that prevent the completion of apoptosis but do not prevent the axon apoptotic signal from activating *c*-jun.


A
axon terminal
axon
cell body
NGF
activated TrkA
retrograde survival signal
c-jun
B
NGF
activated TrkA
retrograde survival signal
axon apoptotic signal
c-jun
Loss of NGF signaling in axons
inactive TrkA
axon apoptotic signal
c-jun
apoptosis
C
inactive TrkA
axon apoptotic signal
c-jun
apoptosis

supports a mechanism in which the delivery of a retrograde apoptotic signal rather than the loss of a retrograde survival signal controls *c*-jun activation.

This study identified two kinase inhibitors, rottlerin and chelerythrine, which prevented the apoptosis of neurons deprived of NGF when applied to distal axons, suggesting that they acted by preventing a required axon apoptotic signaling process. However, it could be alternatively hypothesized that rottlerin and chelerythrine prevent apoptosis when applied to distal axons by generating a retrograde survival signal. Several pieces of evidence argue in favor for the suppression of a retrograde apoptotic signal by rottlerin and chelerythrine rather than the generation of a retrograde survival signal. The data in this study indicate that *c*-jun activation in response to NGF deprivation is due to the delivery of a retrograde apoptotic signal to the cell bodies as opposed to the loss of a retrograde survival signal arriving at the cell bodies. Therefore, the observation that rottlerin and chelerythrine when applied to the distal axons prevent *c*-jun activation suggests that they act by interfering with the retrograde apoptotic signaling pathway occurring in axons. Since several studies have shown that interfering with *c*-jun activation (Eilers et al., 1998; Ham et al., 2000; Virdee et al., 1997) or deleting *c*-jun (Palmada et al., 2002) prevents apoptosis in response to NGF deprivation, it is expected that the ability of rottlerin and chelerythrine to inhibit the axon apoptotic signaling that leads to *c*-jun activation would be sufficient to prevent apoptosis as well. Moreover, rottlerin applied to the distal axons inhibited the activation of GSK3 in the distal axons occurring during NGF deprivation. Experiments with siRNA against GSK3 demonstrate that GSK3 promotes sympathetic neuron apoptosis induced by NGF deprivation. This evidence supports the concept that rottlerin applied to distal axons prevents apoptosis by suppressing the retrograde apoptotic signaling pathway rather than by generating retrograde survival signals.

4.4.1 Implications of the discovery of the retrograde apoptotic signal for previous studies examining NGF retrograde survival signaling

The results of this study indicate that a key mechanism by which NGF on distal axons promotes survival is by suppressing the generation of a retrograde apoptotic signal. Therefore, in all previous studies examining NGF-mediated retrograde survival, it is expected that the suppression of the retrograde apoptotic signal by NGF was a major factor contributing to neuronal survival. However, suppression of the retrograde apoptotic signal is unlikely to be the only mechanism by which NGF acting on axons promotes survival. Indeed, data from the previous chapter of this thesis provide evidence that NGF signaling from distal axons induces the activation of survival signaling molecules, Akt and CREB, in the cell bodies/proximal axons. This suggests that NGF signaling at distal axons also produces retrograde survival signals that mediate the activation of Akt and CREB.

The existence of the axon apoptotic signal is expected to have implications for studies that examine NGF retrograde signaling in compartmented cultures. Since data obtained in this study using 5-compartmented cultures indicate that the axon apoptotic signal may be generated from axon segments containing axon terminals, it is probable that the presence of axon terminals in the compartment containing the cell bodies/proximal axons would lead to the generation of the axon apoptotic signal when NGF is withdrawn from this compartment. The compartmented culture conditions used in this thesis and by other studies performed in the Campenot laboratory result in the majority of axon terminals being localized to the compartment containing the distal axons. This likely explains why *c*-jun is not detectably activated when the cell bodies/proximal axons are deprived of NGF in these cultures, as long as the distal axons are supplied with NGF. In contrast to the compartmented cultures used in this study, other studies have used compartmented cultures in which the majority of the axonal material is localized to the compartment containing the cell bodies/proximal axons, according to visual assessment of the photomicrographs of the cultures provided (Ye et al., 2003). It is plausible that in these cultures there is a significant fraction of the axon terminals in the culture located in the compartment containing the cell bodies/proximal axons. Therefore, when NGF is withdrawn from the cell bodies/proximal axon compartment of such cultures, there may be a strong activation of the axon apoptotic signal and resulting *c*-jun activation in the neuronal cell bodies even when NGF is being supplied to the compartments containing the distal axons. The activation of death signaling in neurons may compromise the ability of some retrograde survival signals to promote survival, since signals may not be equally effective at counteracting the death signal's effects. This may explain the results of Ye et al. (2003) which demonstrated that NGF retrograde survival required the presence of phosphorylated TrkA in the cell bodies/proximal axons. In these cultures, where the death signal may be activated by the withdrawal of NGF from the cell bodies/proximal axons alone, possibly only retrograde signals utilizing phosphorylated TrkA may be effective at promoting survival.

The discovery of the axon apoptotic signal may also offer an explanation for the results of the previous chapter in which K252a applied to either the distal axons or the cell bodies/proximal axons was found to promote the survival of sympathetic neurons completely deprived of NGF. The findings of the present study raise the possibility that K252a may be promoting survival by suppressing the axon apoptotic signal. Although K252a is commonly used as a Trk inhibitor, it has also been found to be a potent inhibitor of MLK3 with an IC_{50} of approximately 5 nM *in vitro* (Roux et al., 2002). Since a major finding of this study is that the axon apoptotic signal led to the activation of *c*-jun, it is probable that the apoptotic signaling pathway lying upstream of *c*-jun is involved in the axon apoptotic signal as well. MLKs are a component of the pathway lying upstream of *c*-jun in sympathetic neurons (Xu et al., 2001). Therefore, K252a's pro-survival effect may be due to inhibition of the MLKs that mediate *c*-jun activation, and the observation that K252a can inhibit apoptosis when applied to the distal axons may signify that MLKs are a component of the axon apoptotic signaling pathway.

4.4.2 Finding the molecular target of rottlerin

Although PKCδ was initially identified as a potential target of rottlerin and chelerythrine which might be involved in the axon apoptotic signaling pathway, experiments with PKCδ siRNA did not demonstrate a requirement for PKCδ in promoting apoptosis induced by NGF deprivation. At present, there is still a possibility that the residual PKCδ protein (< 25%) remaining in PKCδ siRNA-transfected neurons was sufficient for generation of the axon apoptotic signal. However, in light of a recent report (Davies et al., 2000) demonstrating that rottlerin was unable to inhibit PKCδ activity *in vitro* at much higher concentrations of the drug (20 μM) than used in the present study (2.5 μM) argues against the idea that PKCδ is a target of rottlerin inhibition in sympathetic neurons. In a study examining PKC inhibition by chelerythrine, it was found that chelerythrine was ineffective at inhibiting the activity of PKCs isolated from rat brain extracts at concentrations less than 100 μM (Lee et al., 1998), which was well above the concentration of chelerythrine employed in the experiments of this study (10 μM). Thus, it is unlikely that either rottlerin or chelerythrine inhibited PKCδ in this study. In *in vitro* kinase assays, rottlerin (20 μM) was found to have an inhibitory effect on the activity of 13 out of the 24 kinases tested (Davies et al., 2000), which raises the possibility that rottlerin may function as a broad spectrum inhibitor of multiple protein kinases when applied to cells. Based on these data, it is plausible that rottlerin may act on multiple targets in sympathetic neurons to inhibit the axon apoptotic signal. Therefore, although rottlerin and chelerythrine have been useful in helping to demonstrate the existence of the axon apoptotic signal, identifying a specific molecular target for its actions in the near future may prove to be a difficult task.

4.4.3 The role of GSK3 in sympathetic neuron apoptosis

The results of this study demonstrate that GSK3 promotes sympathetic neuron apoptosis in response to *NGF* deprivation. In contrast, a previous study found that transfection of sympathetic neurons with either binding partners of GSK3β that inhibit its activity (GBP and Frat1) or a GSK3β kinase-dead mutant did not rescue neurons from *NGF* deprivation-induced apoptosis (Crowder and Freeman, 2000). One possible reason for why a pro-apoptotic role for GSK3 could not be demonstrated in this study is that GSK3 inhibitors such as Frat1 and GBP have been shown to prevent GSK3β from binding and phosphorylating some targets such as axin, β-catenin, and Tau without affecting its ability to phosphorylate other targets such as glycogen synthase (Culbert et al., 2001; Thomas et al., 1999). Therefore, in those experiments, GSK3 may have retained

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its ability to activate pro-apoptotic targets. It is also possible that the inhibitors used were not effective at inhibiting the GSK3α isoform, which we found was also involved in apoptosis. Therefore, the technique used to prevent GSK3 function in this study, via reduction of the total GSK3 protein levels, may be more suited for determining if GSK3 is required for apoptosis.

In the experiments with GSK3 siRNA in this study, it was found that reduction of 75% of the total GSK3 protein resulted in only a 27% decrease in apoptosis in response to NGF deprivation. A possible reason for why reduction of 75% of GSK3 protein did not produce a greater protective effect may be that the residual GSK3 in a significant portion of transfected neurons was sufficient to generate apoptotic signals. Another possibility is that because GSK3 has been shown to be involved in other neuronal processes such as axon and dendrite formation and growth (Garrido et al., 2007; Kim et al., 2006; Yoshimura et al., 2006), the impairment of these processes by the removal of GSK3 could have negative implications for neuronal survival.

The observation that both GSK3 isoforms had to be knocked down in order to observe an anti-apoptotic effect suggests that GSK3α and GSK3β have an overlapping function in the apoptotic pathway of sympathetic neurons. This is plausible since GSK3α and GSK3β isoforms are closely related, with the rat protein sequences sharing 85% identity overall and 98% identity within the catalytic domain (Woodgett, 1990). Functional redundancy of GSK3α and GSK3β has been demonstrated for the Wnt signaling pathway (Doble et al., 2007). However, GSK3α and GSK3β also possess distinct functions as evidenced by unique phenotypes found between GSK3α and GSK3β knockout mice (Hoeflich et al., 2000; Kerkela et al., 2008; MacAulay et al., 2007).

One important question is how GSK3 fits into the apoptotic pathway of sympathetic neurons. GSK3 has been implicated in apoptosis in response to a broad range of stimuli (reviewed by Beurel and Jope, 2006). Previous studies investigating GSK3's pro-apoptotic activity may offer clues about how GSK3 is activated and promotes apoptosis during NGF deprivation. A major candidate for regulating the activation of GSK3 is Akt. Akt inhibits the activity of GSK3s by phosphorylating them at Ser9/Ser21 (GSK3β/GSK3α) (Cross et al., 1995). Since NGF supplied to distal axons induces the local activation of Akt (Kuruvilla et al., 2000; MacInnis et al., 2003; Ye et al., 2003, and Figure 3-1), it is possible that Akt-mediated phosphorylation of GSK3 suppresses axon apoptotic signaling. Loss of NGF signaling at the distal axons and inhibitory phosphorylation of GSK3 by Akt would allow for GSK3 activation. GSK3 kinase activity would mediate the phosphorylation of its pro-apoptotic targets. Targets of GSK3 that have been implicated in regulating sympathetic neuron apoptosis include MLK3 (Mishra et al., 2007), p53 (Watcharasit et al., 2003), Bax (Linseman et al., 2004), and CREB (Bullock and Habener, 1998). In regard to substrates that may be involved in the axon apoptotic signaling pathway identified in this study, MLK3 is the prime candidate because it is an upstream component of the *c*-jun pathway. However, it cannot be ruled out at this time that GSK3 may act at multiple points during apoptosis.

4.4.4 Possible components of a retrograde apoptotic signal

The apoptotic signal generated in axons must be retrogradely transmitted to the cell bodies in order to mediate activation of *c*-jun. What is the possible identity of the carrier of the retrograde apoptotic signal? A reasonable hypothesis is that the carrier will be found in the characterized JNK signaling pathway in sympathetic neurons that lies upstream of *c*-jun activation. A characteristic that may help to identify carriers of retrograde signals is that the signaling molecules are likely activated in both the distal axons and the cell bodies/proximal axons. As preliminary evidence supporting that components of the JNK pathway may carry the retrograde apoptotic signal, JNKs and MKK4 were found to be activated in both the distal axons and the cell bodies/proximal axons in response to NGF deprivation. There is evidence to suggest that several members of the JNK pathway (MLK3, MKK4/7, JNKs) may be held together in signaling complexes by the scaffold proteins, POSH and JIP-1 (Kukekov et al., 2006; Xu et al., 2003). POSH function has been implicated in the activation of *c*-jun and the promotion of apoptosis in sympathetic neurons induced by NGF deprivation (Xu et al., 2003). Therefore, it is plausible that the retrograde apoptotic signal delivered to the cell bodies may be an apoptotic signaling complex held together by POSH and JIP-1.

The observation that application of the GSK3 inhibitor, LiCl, to either the distal axons or the cell bodies/proximal axons, prevented *c*-jun activation in response to NGF deprivation may indicate that GSK3 is a carrier of the retrograde apoptotic signal (shown schematically in Figure 4-14A). There is evidence in PC12 cells demonstrating that MLK3, one of the components of the POSH/JIP-1 apoptotic signaling complex, is a target of GSK3β kinase activity (Mishra et al., 2007). Therefore, activation of MLK3 by GSK3β, and potentially by GSK3α, if transported together, may help to maintain the retrograde apoptotic signal as it travels through the axons. It also remains a possibility that GSK3 could be retrogradely transported without other molecules as an apoptotic signal (Figure 4-14B), or that GSK3 is not retrogradely transported but is activated in

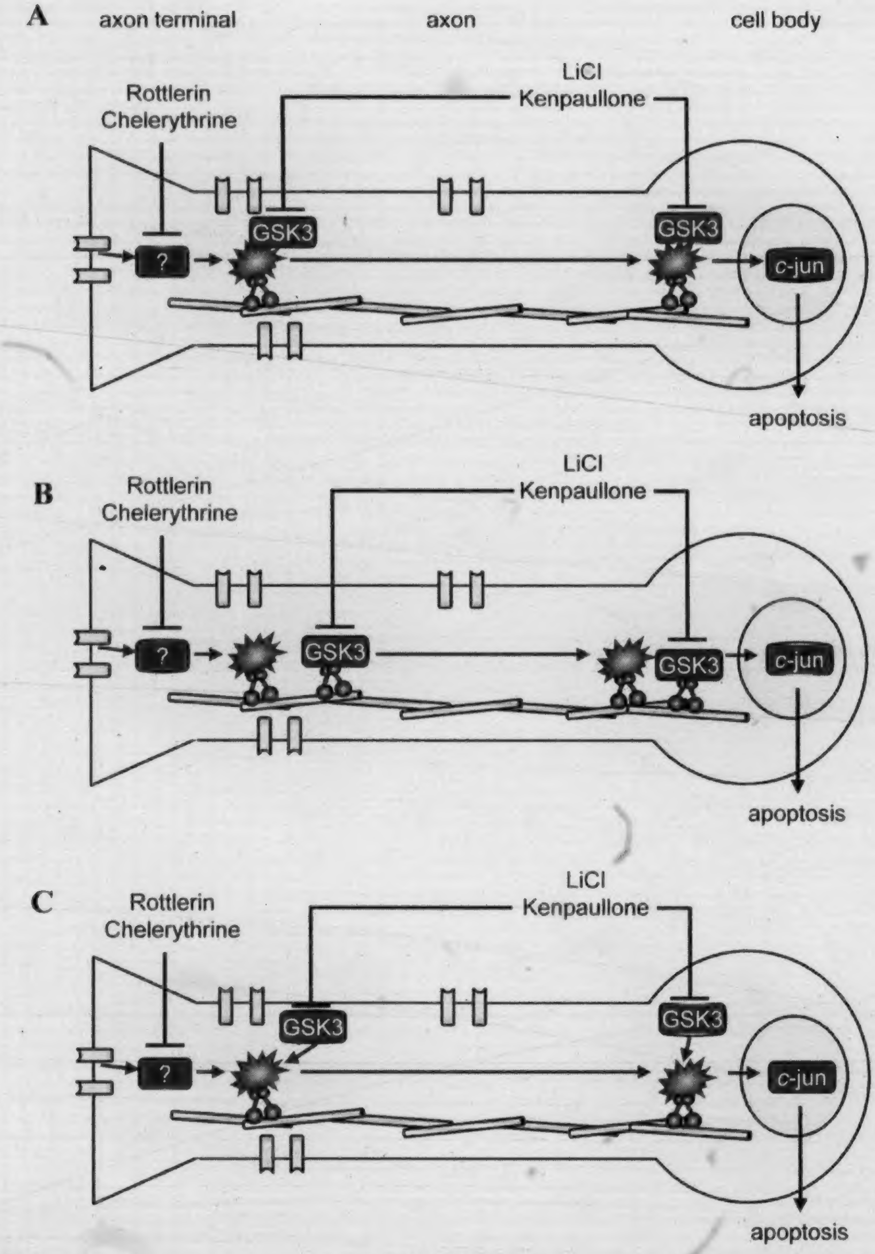
A
axon terminal
axon
cell body
LiCl
Kenpaullone
Rottlerin
Chelerythrine
GSK3
?
GSK3
c-jun
apoptosis
B
LiCl
Kenpaullone
Rottlerin
Chelerythrine
?
GSK3
GSK3
c-jun
apoptosis
C
LiCl
Kenpaullone
Rottlerin
Chelerythrine
GSK3
GSK3
?
c-jun
apoptosis

Figure 4-14. Potential mechanisms for how GSK3 promotes axon apoptotic signaling. A) GSK3 (α and β isoforms) is a component of the carrier of the retrograde apoptotic signal that is transmitted from the axons to the cell bodies. B) GSK3 is one of multiple retrograde apoptotic signal carriers that are transmitted from the axons to the cell bodies. C) GSK3 is not itself retrogradely transported but is activated in both the distal axons and cell bodies in response to NGF deprivation to promote the functioning of the retrograde apoptotic signal.

both the distal axons and cell bodies/proximal axons to promote the functioning of the retrograde apoptotic signal (Figure 4-14C).

How would a retrograde apoptotic signal that may be composed of a POSH/JIP-1 apoptotic signaling complex and/or GSK3 be retrogradely transported? Experiments with colchicine indicate that the transmission of the retrograde apoptotic signal depends on microtubule-based transport, suggesting that linking to retrogradely moving molecular motors may retrogradely transport the signal. One possibility is that a component of the retrograde apoptotic signal may be able to directly bind to retrograde motors (shown schematically in Figure 4-15A). Members of the JIP family directly bind to anterograde motors (Bowman et al., 2000; Kelkar et al., 2005; Verhey et al., 2001) and retrograde motors (Cavalli et al., 2005) to facilitate the transport of JIP-bound cargo. Although JIP-1 has not been specifically demonstrated to bind directly to retrograde motors, a study in *Drosophila* has shown that mutation of the homolog of JIP-1 results in impaired retrograde transport of vesicles and mitochondria, suggesting that JIP-1 is involved in linking retrograde motors to cargo (Horiuchi et al., 2005). Another way by which a retrograde apoptotic signal may link to retrograde motors is through association with retrogradely transported endosomes (Figure 4-15B). One way this may be accomplished is through the recruitment of the JIP-1/POSH apoptotic signaling complex by Rac1. Rac1 is post-translationally modified by geranylgeranylation (Menard et al., 1992), which facilitates its association with membranes. Since POSH binds activated Rac1 (Tapon et al., 1998), this interaction following NGF deprivation may lead to the recruitment of the POSH/JIP-1 complex to the membrane which could then be internalized and trafficked to endosomes for retrograde transport.

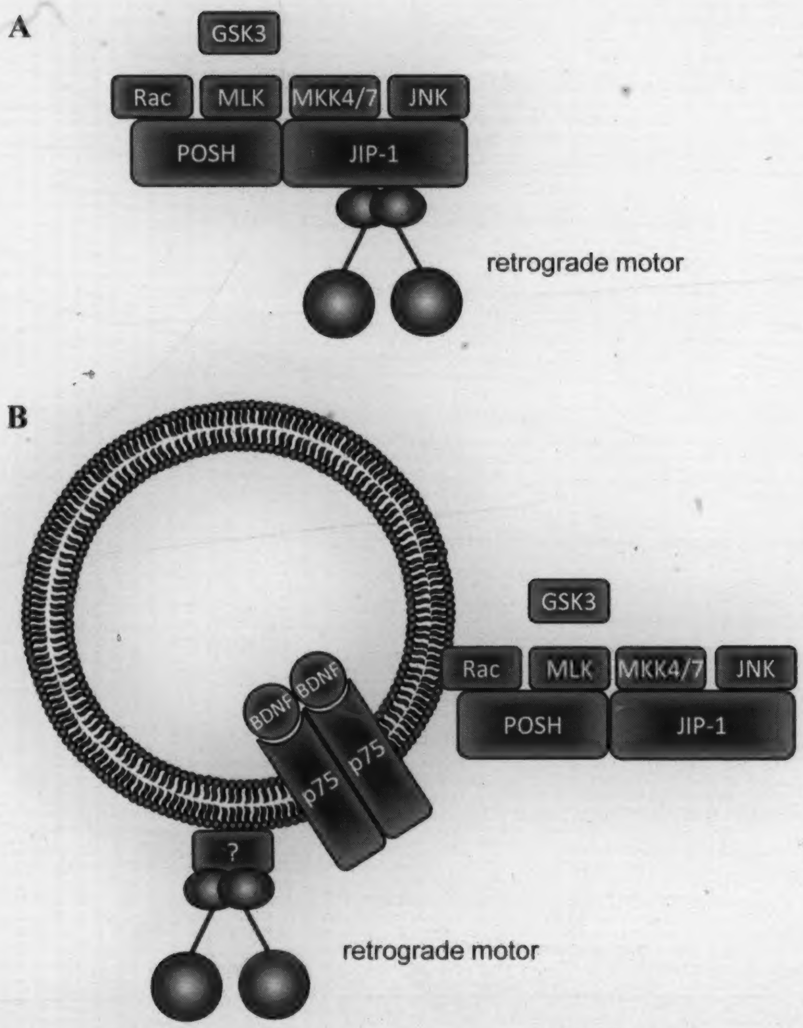
A
GSK3
Rac
MLK
MKK4/7
JNK
POSH
JIP-1
retrograde motor
B
GSK3
Rac
MLK
MKK4/7
JNK
POSH
JIP-1
BDNF
BDNF
p75
p75
?
retrograde motor

Figure 4-15. Potential carriers of the retrograde apoptotic signal. A) An apoptotic signaling complex held together by the scaffold proteins, POSH and JIP-1, may be able to directly associate with retrograde motors via JIP-1. GSK3 may be able to interact with the scaffold, although a specific interaction has not been identified. B) The scaffolded apoptotic signaling complex may be able to associate with endosomes which are potentially generated by internalization of p75NTR following BDNF stimulation. The apoptotic signaling complex may be recruited to endosomes through the ability of the prenylated (geranylgeranyl) Rac to interact with membranes. GSK3 may also be able to interact with the scaffold or endosomes, although a specific interaction has not been identified. Endosomes must somehow be linked by an adaptor (?) to retrograde motors. Since JIP-1 is able to link cargo to retrograde motors as described in (A), it may also be able to link endosomes to retrograde motors as well.

If endosomes are involved in trafficking the retrograde apoptotic signal, it is possible that the internalization of receptors during NGF deprivation and their subsequent trafficking to endosomes might facilitate the retrograde apoptotic signal by providing a source of endosomes for retrograde transport (Figure 4-15B). A candidate receptor for generating retrogradely transported endosomes may be p75NTR, a receptor implicated in promoting sympathetic neuron apoptosis. p75NTR was shown to promote apoptosis after addition of neurotrophins other than NGF, such as BDNF, proBDNF, proNGF, or NT-4 (Bamji et al., 1998; Deppmann et al., 2008; Kenchappa et al., 2006; Nykjaer et al., 2004). Endogenously synthesized and secreted BDNF (Causing et al., 1997; Kohn et al., 1999; Singh et al., 2008) in cultured sympathetic neurons may activate p75NTR and induce its internalization as has been demonstrated in sympathetic neurons by Hibbert et al. (2006). Therefore, it is plausible that p75NTR might participate in the axon apoptotic signal perhaps by being retrogradely transported in endosomes and carrying the retrograde apoptotic signal along with it. The finding that Rac has been implicated in p75NTR signaling (Harrington et al., 2002) offers a possible route for Rac activation in axons following NGF deprivation.

One very interesting observation in the present study is that the retrograde apoptotic signal is capable of activating *c*-jun in the cell body even in the presence of NGF-generated survival signaling at the cell bodies/proximal axons. The activation of *c*-jun under these conditions was still observed even after 24 h. One possible explanation for these results is that NGF provided to the cell bodies/proximal axons cannot turn off *c*-jun activation because it is less effective at generating survival signals than NGF supplied to distal axons. However, NGF supplied to the cell bodies/proximal axons was shown to promote changes in gene expression that were 2- to 3- fold higher than when NGF was applied to the distal axons of compartmented cultured sympathetic neurons (Toma et al., 1997). Also, NGF supplied to the cell bodies/proximal axons supported survival just as well as NGF supplied to the distal axons as assessed by examining nuclear morphology (Ye et al., 2003), a result that was confirmed in the present study. These findings argue against the hypothesis that NGF supplied to the cell bodies/proximal axons cannot turn off *c*-jun activation because it does not produce effective amounts of survival signals.

An alternative hypothesis for the results presented is that NGF signaling prevents the activation of the apoptotic signal in the axons, but once it is generated, NGF signaling cannot effectively deactivate any of its components that lead to *c*-jun activation. Thus, during its journey through the axons and after arriving at the cell bodies the retrograde apoptotic signal is able to maintain its activation. One mechanism that may protect the retrograde apoptotic signal from deactivation may be mediated by scaffold proteins such as JIP-1 and POSH. Scaffold proteins bind multiple components of the same pathway, thereby bringing together upstream kinases and downstream substrates in close proximity (reviewed by Dard and Peter, 2006). This not only facilitates the initial activation of the bound signaling molecules but also maintains close physical proximity of molecules so that when a kinase becomes deactivated it can be readily reactivated by its upstream kinase. Scaffold proteins have also been shown to target to specific cellular sites via adaptors that may help to segregate the scaffold and its bound components from areas with high concentrations of deactivating molecules (Harding et al., 2005). Another means of maintaining the activation of the retrograde apoptotic signal may be through the ability of MLK3 to undergo homodimerization and autophosphorylation, thereby sustaining its own activation (Leung and Lassam, 1998). Yet another mechanism for preventing deactivation of the retrograde apoptotic signal is that upon arriving at the cell bodies, the retrograde apoptotic signal or the molecules activated downstream of this signal are shuttled to specific subcellular locations, possibly by the targeting of scaffold proteins. Subcellular localization may facilitate downstream signaling or provide segregation from high concentrations of deactivating molecules. For example, JNKs were found to localize to nuclei of cerebellar granule neurons, and the activity of nuclear-localized JNKs were critical for promoting apoptosis of these neurons in response to trophic withdrawal (Bjorkblom et al., 2008). If the retrograde apoptotic signal in sympathetic neurons mediates the nuclear localization of JNKs, the JNKs would be ideally located to keep *c*-jun activated. In addition, it has been found that the scaffolds POSH and JIP-1 accumulate to perinuclear locations under apoptotic conditions in PC12 cells (Kukekov et al., 2006), which raises the interesting possibility that POSH and JIP could be used to deliver apoptotic signaling molecules of the JNK pathway to the optimal location for facilitating and maintaining *c*-jun activation.

4.4.5 The possible role of axon terminals in generating the axon apoptotic signal

Evidence presented in this study indicates that rottlerin protects neurons from apoptosis specifically when applied to the axon segments containing the axon terminals (Figure 4-6), and it is hypothesized that the axon apoptotic signal may be generated specifically at axon terminals. Restricting the generation of the apoptotic signal to axon terminals makes sense if one considers the environment of sympathetic neurons during development. *In vivo*, the NGF that is secreted in limiting amounts from target cells is only concentrated at the nearby axon terminals of the innervating sympathetic neurons.

Thus, it is improbable for the axon apoptotic signal to be generated at any point along the axon since a large majority of the axon connecting the axon terminals to the cell body is normally not in contact with NGF. Restricting the generation of the axon apoptotic signal to axon terminals would allow the signal to be used as a reliable indicator that a nerve terminal is not receiving sufficient NGF signaling due to ineffective or inappropriate targeting.

If axon terminals are the initiation sites for the retrograde apoptotic signal, then it is possible that molecules involved in the suppression and generation of the apoptotic signal will be localized to these sites. Several studies have provided evidence that potential regulatory components of the axon apoptotic signaling pathway are found at growth cones. For instance, the morphology and directional movement of growth cones are dependent on the presence of NGF (Connolly et al., 1987; Gundersen and Barrett, 1979; Seeley and Greene, 1983), suggesting that NGF signaling occurs in growth cones. Additionally, it has been demonstrated in DRG sensory neurons that GSK3β localizes to growth cones and that a signaling cascade involving NGF-mediated activation of PI3K and Akt, which results in the inhibition of GSK3, promotes axon extension (Zhou et al., 2004). Rac has also been implicated in functions at the growth cone (Dickson, 2001; Grabham et al., 2003; Jurney et al., 2002). Therefore, several of the proteins that are already localized at the growth cone for mediating other neuronal functions such as axon growth may also be used to support the generation of the axon apoptotic signal in the absence of NGF. Other supporting evidence that molecules potentially involved in generating the axon apoptotic signal are found in axon terminals is the observation that JIP-1 is concentrated at the tips of axon-like extensions of two neuronal cell line models, PC12 cells (Kukekov et al., 2006) and Cath.a-differentiated (CAD) cells (Verhey et al., 2001). In CAD cells, it was found that JIP-1co-immunoprecipated with the MLK family member, DLK, and that DLK was localized to the tips of axon-like extensions (Verhey et al., 2001).

It is also possible that not all signaling components of the axon apoptotic signaling pathway are localized to axon terminals but are instead distributed throughout the axon. If this were the case, it would be expected that the molecules are spatially configured in the axon terminals in a manner that optimizes their activation, perhaps by being in close association with other pathway components.

4.4.6 Biological significance of the axon apoptotic signal

The results of this study demonstrate that NGF-deprived axons generate an apoptotic signal that is retrogradely transported to the cell bodies to initiate apoptosis. What would be the purpose of having such a signaling mechanism for regulating survival? One possibility is that it would allow a neuron to compare signals coming from all terminals: terminals that have connected to their target and are successfully competing for NGF, and terminals that have not connected to their targets or are ineffectively competing for NGF. Terminals receiving NGF would generate retrograde survival signals and terminals receiving insufficient NGF would generate retrograde apoptotic signals instead. Both survival and apoptotic signals would be delivered to the cell body and survival of the neuron would depend on the relative magnitude of these signals. The experiments of this study in which NGF is supplied to the cell bodies/proximal axons but withdrawn from the distal axons are a demonstration that both survival signals and apoptotic signals can co-exist in the cell body (Figure 4-1A). Thus, the survival of the neuron could be based on how well a neuron was connected to its target overall, as communicated to the cell bodies by the magnitude of apoptotic versus survival signals. Neurons having the requisite proportion of successfully connected terminals would be selected for survival. A possible *in vivo* example of neuron survival regulated by the balance of retrograde survival signals and retrograde apoptotic signals is the sympathetic neurons of the superior cervical ganglia innervating the iris. The developing sympathetic neurons that project axons to the iris, send axons branches to both the anterior and posterior compartment of the iris. Following the period of developmental cell death in rats, 40% of the sympathetic neurons projecting to the iris have branches innervating both compartments (Vidovic and Hill, 1988) and for mice, 78% of the neurons projecting to the iris innervate both compartments (Singh et al., 2008). It is interesting to speculate that a high percentage of neurons innervate both compartments because the generation of the retrograde apoptotic signal in neurons that innervate only one compartment promotes their apoptosis.

Another possible function for the axon apoptotic signal is that it could be used to prime neurons for apoptosis so that they may rapidly undergo apoptosis once NGF signaling at the cell bodies is depleted. Upon examining the rates of retrograde trafficking of NGF in compartmented cultures of sympathetic neurons, it is observed that when 200 ng/ml NGF is applied to the distal axons, it is transported and degraded in the cell bodies with a half-life of 6 h (Ure and Campenot, 1997). Based on these results, it is expected that even after 6 h of NGF deprivation, there would still be a considerable fraction of intact NGF available for signaling within the cell bodies. If the axon apoptotic signal did not occur and, therefore apoptotic signaling could not be initiated until enough NGF had been degraded for survival signaling to drop to insufficient levels, the time required for a neuron to undergo apoptosis could likely be significantly longer. A mechanism such as the axon apoptotic signal may allow the apoptotic process to get a head start, so that it can proceed quickly once NGF signaling becomes insufficient to halt its progression.

Another interesting possibility is raised by the study of Deppmann et al. (2008). They did a comparative analysis of the gene expression patterns of sympathetic neurons isolated from mice with intact (NGF-secreting) or ablated target cells and found several differences in expression of several genes, including TrkA. The observation that loss of NGF signaling resulted in reduced TrkA expression was used to make predictions about how NGF-regulated changes in TrkA expression could serve as a factor for neuronal competition during development: neurons receiving strong NGF signals would continue to express large amounts of TrkA that would facilitate more NGF signaling whereas neurons receiving weak NGF signals would express less TrkA, making it increasingly difficult for these neurons to engage in NGF signaling. Perhaps the axon apoptotic signal also functions to enhance neuronal competition during development. The axon apoptotic signal may induce downstream signaling or changes in gene expression that impede the generation of retrograde survival signals or render the neuron less responsive to survival signals. Neurons that generate strong apoptotic signals at the start of the period of NGF-dependence would find it increasingly difficult to maintain enough survival signaling and would be quickly eliminated, whereas neurons able to generate strong NGF signaling at the outset of NGF-dependence would have a greater chance of surviving.

The axon apoptotic signal has the potential to be a widespread mechanism in neuronal development since target-derived neurotrophic factors, such as neurotrophins, regulate the survival of many neuron populations (Burke, 2006; Henderson et al., 1994; Huang and Reichardt, 2001; Lahteenmaki et al., 2007; Oppenheim et al., 1995; Whitehead et al., 2005). The axon apoptotic signal may continue to negatively regulate neuron survival after development. Diminished neurotrophic factor signaling has been implicated as a contributing factor for the neurodegeneration associated with diseases such as Alzheimer's disease, Huntington's disease, and Parkinson's disease (reviewed by Fumagalli et al., 2008; Williams et al., 2006). Generation of the apoptotic signal due to diminished neurotrophin signaling may promote the apoptosis of diseased neurons. In support of a possible role for the axon apoptotic signal in neurodegenerative diseases, activation of the JNK pathway and *c*-jun have been demonstrated in animal models of Parkinson's disease (Saporito et al., 2000; Willesen et al., 2002). JNK activation has also been demonstrated in an animal model of Alzheimer's disease (Shoji et al., 2000), and JNK phosphorlyation (Shoji et al., 2000) and *c*-jun phosphorylation (Thakur et al., 2007) are observed in affected brain regions of Alzheimer's disease patients, post-mortem. GSK3 activation is also implicated in Alzheimer's disease pathology (reviewed by Hernandez and Avila, 2008).

It is reasonable to hypothesize that the axon apoptotic signal may also be induced by other stimuli besides neurotrophin withdrawal. It is also possible that the components of the axon apoptotic signaling pathway may be altered following development to modulate its downstream effects. In fact, there may also be as of yet unidentified retrograde apoptotic signals that operate during development and in adulthood to regulate survival. It will be important to assess if retrograde death signaling is occurring in other apoptotic models and what stimuli activate them. The potential importance of retrograde death signals in axons will certainly affect the way we examine the life and death of neurons in future research.

CHAPTER 5:

PERSPECTIVES

5.1 Synopsis

In this thesis, evidence is provided for two NGF-dependent retrograde signaling mechanisms that regulate sympathetic neuron survival. In the first mechanism, NGF supplied to the distal axons produces a retrograde survival signal that does not require the presence of phosphorylated TrkA in the cell bodies to mediate its survival-promoting effects. In the second mechanism, NGF supplied to the distal axons suppresses a retrograde apoptotic signal that is referred to as the axon apoptotic signal. When NGF is withdrawn from the distal axons, the axon apoptotic signal is generated and transmitted retrogradely to the cell bodies to induce apoptotic signaling. The existence of multiple retrograde signals for mediating survival warrants continued investigation not only because it will contribute to our understanding of neuronal development but also because these signals may continue to be used in adulthood by the neuron to continue to respond to its environment during events such as neurotrauma and neurodegenerative diseases.

5.2 Avenues for exploration in future studies

The molecular components underlying the retrograde signals examined in this thesis are just beginning to be characterized. For the retrograde survival signal that does not require TrkA phosphorylation in the cell bodies, it was found that Akt is phosphorylated in the distal axons and that both Akt and CREB are phosphorylated in the cell bodies/proximal axons. For the axon apoptotic signal, it has been demonstrated that its generation leads to nuclear accumulation of phosphorylated *c*-jun in the cell bodies/proximal axons. There is also evidence provided with inhibitors for GSK3 that demonstrate that GSK3 activity is required in both the distal axons and the cell bodies/proximal axons for the axon apoptotic signal to mediate both *c*-jun phosphorylation and apoptosis. Several signaling molecules upstream of *c*-jun including MKK7, MKK4, and JNK are phosphorylated in the distal axons when apoptosis is initiated, and both MKK4 and JNK are also phosphorylated in the cell bodies/proximal axons under these conditions. There is also evidence obtained with a Rac inhibitor that suggests that Rac activity in the distal axons is required by the axon apoptotic signal to produce *c*-jun phosphorylation in the cell bodies/proximal axons and promote apoptosis. Guided by this molecular framework and the data provided by previous studies, which have already identified numerous molecules involved in NGF survival signaling and apoptotic signaling induced by NGF deprivation, the molecular details of these retrograde signals can begin to be elucidated.

5.2.1 What are the carriers of retrograde signals?

To identify the carriers of retrograde signals it may be helpful to first define the characteristics that such a carrier would possess. It is probable that a carrier would be activated in the distal axons and then transmitted retrogradely through the axons in its activated form so that when it arrived at the cell bodies it could initiate further downstream signaling. Therefore, a molecule that functions as a carrier should be observed moving retrogradely in its active form through the axons. In addition, the activation of the retrograde carrier is expected to be required in both the distal axons and the cell bodies/proximal axons in order for the signal to be preserved throughout the journey and arrive at the cell bodies still capable of inducing downstream signals. Finally, it is likely that the retrograde carrier will be identified in the cell bodies as the most upstream signaling molecule activated in its pathway, since it will be the initiation point of further downstream signaling.

Given the above criteria, can potential candidates for carrying the retrograde signal be identified based on the data presented in this thesis? For the retrograde survival signal that does not require TrkA phosphorylation at the cell bodies it is demonstrated that Akt is phosphorylated in both the distal axons and the cell bodies/proximal axons, which is consistent with a parameter described for a retrograde carrier. However, there is no reason to exclude the possibility that carriers of retrograde signals may be composed of more the one signaling molecule. For example, the scaffolding protein, APPL1, which binds Akt, PI3K subunits and TrkA (Lin et al., 2006), may also participate in carrying the retrograde survival signal by tethering Akt to retrogradely transported TrkA. For the axon apoptotic signal, evidence with inhibitors of GSK3 suggest that GSK3 activity is required in both the distal axons and cell bodies/proximal axons for mediating signaling events in the cell bodies and for promoting apoptosis, which make it a potential retrograde signal carrier. There is also evidence that MKK4 and JNKs are phosphorylated in both the distal axons and the cell bodies/proximal axons in response to NGF deprivation, which also makes them candidates for retrograde carriers. In the case of MKK4 and JNK, there are studies suggesting that these proteins may be associated with other JNK pathway members in an apoptotic signaling complex held together by the scaffold proteins POSH and JIP-1 (Kukekov et al., 2006; Xu et al., 2003). There is also the possibility that the retrograde apoptotic signal is trafficked by associating with endosomes. These endosomes may be created by the internalization of p75NTR following binding to endogenously secreted BDNF (Hibbert et al., 2006).

What experiments can be done to determine if potential candidates are retrograde carriers? An experiment can be devised that makes use of the five-compartmented culture. In these cultures, signals that are generated in the distal axons and travelling retrogradely will have to pass through the intermediate compartment prior to their arrival at the cell bodies/proximal axons. Thus, the appearance of activated retrograde carriers should be detectable in the intermediate compartments and at an earlier time point than its appearance in the cell bodies/proximal axons. The caveat of this experiment is that it cannot be resolved if the molecules being examined are the retrograde carriers or if they are being phosphorylated by the carrier as it travels past them down the axon. If inhibitors of the signaling molecules of interest are available they could be used to help verify retrograde carriers, since inhibiting the carrier at any point along the axons or at the cell bodies would block its transmission. However, if retrograde signals are composed of multiple signaling molecules of the same pathway as described for scaffold complexes, it is possible that there is a mechanism for the signal's reactivation once it passes through the region of inhibition. Therefore, alternative assays should be explored that involve techniques in which proteins can be labeled in the distal axons and then monitored for their retrograde transport and activation state. One technique that may be amenable to compartmented culture studies involves introducing gene constructs into sympathetic neurons that would express the protein of interest fused to a small molecule SNAP-tag (Keppler et al., 2003) or Halotag (Los et al., 2008) that allows for the covalent attachment of a variety of cell permeable probes (fluorescent molecules, biotin). In compartmented cultures, these probes could be selectively added to the distal axons and then used to monitor retrograde accumulation of the tagged protein in the cell bodies/proximal axons. Tagged protein could also be purified for further biochemical analysis of its activation state. Creating tagged fusion proteins of retrograde motor components or endosomal markers may also be a beneficial study, since the purification of the tagged proteins may also pull-down associated molecules or endosomes. A less specific method for detecting the retrograde transport of proteins of interest would be to label all proteins in the distal axons using cell-permeable biotin crosslinkers, and then to purify the retrogradely transported labeled material for analysis by immunoprecipitation or for *in vitro* activity assays.

To determine if p75NTR-containing endosomes are involved in carrying the retrograde apoptotic signal, a preliminary experiment can be performed using a function blocking antibody against p75NTR (REX) that prevents p75NTR internalization and signaling (Weskamp and Reichardt, 1991).

5.2.2 Where are the sites for the generation of the axon apoptotic signal?

This thesis provides evidence indicating that the axon apoptotic signal is generated from axon segments containing the axon terminals. This raises the possibility that axon terminals may be the sites from which the axon apoptotic signal is generated. One way to achieve such a signal would be to localize the signaling components required for initiating the axon apoptotic signal specifically to the axon terminals. To determine if this is the case, an assessment of the localization of potential components of the axon apoptotic signal should be carried out. A method for determining localization is to probe fixed cultures with antibodies against proteins of interest and determine their distribution in axons by immunofluorescence confocal microscopy. From these studies one or more apoptotic molecules may be identified to preferentially localize to axon terminal sites.

5.2.3 Can axon apoptotic signals and retrograde survival signals be carried down the axon and arrive at the cell bodies at the same time?

One issue that needs to be further examined is whether or not the axon apoptotic signal can be transmitted through the axon even when NGF survival signals are also present in the axons. Evidence presented in this thesis demonstrates that the axon apoptotic signal continues to produce apoptotic signaling even when the compartment containing the proximal axons and cell bodies is supplied with NGF. This means that the axon apoptotic signal can travel through NGF-supplied proximal axons before arriving at the cell bodies without undergoing complete loss of the signal. However, it is unknown if the axon apoptotic signal could travel longer distances in the presence of competing NGF signaling. The 5-compartmented culture would be useful for examining this issue since NGF can be supplied to the intermediate axons and the cell bodies/proximal axons, thereby lengthening the distance the apoptotic signals generated in the distal axons must travel before reaching the cell bodies. In this experiment, the accumulation of nuclear phosphorylated *c*-jun would provide a valuable marker for the effective transmission of the apoptotic signal.

A hypothesis offered for the purpose of the axon apoptotic signal *in vivo* is that it allows the cell body to receive signals from both properly connected terminals and improperly/ineffectively connected terminals. Is there a method for producing such a scenario *in vitro* so that this hypothesis can be tested? There may be a way of manipulating the culture conditions of 5-compartmented cultures to achieve the desired set-up. For the experiment involving 5-compartmented cultures in this thesis, the NGF was removed from the intermediate axons and cell bodies/proximal axons for 2 days prior to the experiment, to cause degeneration of the axons with terminals in these compartments. During this period, the distal axons continued to receive NGF, and thus the culture conditions selected for neurons with axons that terminated in only the distal compartments. However, if alternatively, 50 ng/ml NGF is supplied to both the intermediate compartments and the distal compartments for a period prior to experiments, it is expected that these conditions will promote axon branching and axon growth within these compartments. This process would likely result in the presence of axon terminals in the distal axon compartments as well as an increased number of axon terminals in the compartment containing the intermediate axons. The distal axons or the intermediate axons could then be supplied with NGF while depriving the other compartments of NGF, and the cell bodies/proximal axons would be monitored for both evidence of retrograde apoptotic signaling (*c*-jun phosphorylation) and retrograde survival signaling (Akt and CREB phosphorylation).

5.2.4 Determining if the axon apoptotic signal operates *in vivo* during development

During development, approximately half the sympathetic neurons of the superior cervical ganglia are eliminated between postnatal day 1 and 15 (Bamji et al., 1998). Sympathetic neurons are dependent on NGF for survival during this period (Coughlin and Collins, 1985; Kessler and Black, 1980), which suggests that the loss of sympathetic neurons seen during this stage of development is due to apoptosis caused by insufficient NGF signaling. Thus, the axon apoptotic signal may be operating during this time. If it is hypothesized that the neuron cell body receives both retrograde survival signals and retrograde apoptotic signals to regulate its survival, then it is expected that a portion of sympathetic neurons in the superior cervical ganglia (SCG) will contain markers for both apoptotic signals and survival signals in their cell bodies. SCGs could be fixed between P1 and P15 and then immunostained for both phosphorylated *c*-jun and Akt. For comparison, the SCGs from rats that have been systemically administered either NGF (Levi-Montalcini and Booker, 1960b) or anti-NGF (Cohen, 1960; Levi-Montalcini and Booker, 1960a) would be examined for the same markers.

Another experiment that may be performed to examine if the axon apoptotic signal is operating during development involves generating the axon apoptotic signal by neutralizing the NGF at an innervation target of sympathetic neurons such as the iris. NGF actions could be neutralized by local injection of NGF antibody into the iris. The iris innervating neurons could be labeled with a retrograde tracer to identify the cell bodies in the SCG. The phosphorylation state of *c*-jun in the cell bodies of the labeled neurons would be assessed. To prevent the neurons from undergoing apoptosis, NGF can be injected subcutaneously near the SCG (Schwab and Thoenen, 1975) to mediate the generation of survival signals at the cell bodies of the sympathetic neurons.

5.2.5 How do survival signals and apoptotic signals at the cell bodies interact to regulate neuronal survival?

The experiments in this thesis in which the distal axons are deprived of NGF but the cell bodies/proximal axons are supplied with NGF create a situation in which apoptotic signals and survival signals are operating in the cell bodies/proximal axons at the same time. This allows for a unique opportunity to examine where survival signaling pathways and apoptotic signaling pathways intersect. According to the data presented in this thesis, the nuclear accumulation of phosphorylated *c*-jun persists even after the distal axons have been deprived of NGF for 24 h. However, the neurons do not complete the apoptotic program when NGF is supplied to the cell bodies/proximal axons as evidenced by the lack of nuclear condensation after 72 h of treatment. How does NGF signaling prevent the progression of apoptosis in these neurons? To answer this question an analysis of signaling events downstream of *c*-jun needs to be performed in order to determine the extent of activation of the apoptotic program. For instance, it can be assayed whether nuclear phosphorylated *c*-jun is able to promote pro-apoptotic gene expression in the presence of NGF survival signals at the cell bodies/proximal axons. There is evidence that the regulation of one of *c*-jun's gene targets, Bim, requires the concurrent binding of two other transcription factors, Myb and FkHRL1/Foxo3a at the Bim promoter (Biswas et al., 2007). It is possible that NGF signaling prevents the activation of Myb and FkHRL1/Foxo3a, thereby blocking *c*-jun-dependent gene expression. It will be interesting to determine if this is the case, or if the axon apoptotic signal can advance the apoptotic program to further downstream events, before being blocked by NGF-mediated signals.

5.2.6 Does the axon apoptotic signal continue to operate after development?

The axon apoptotic signal identified in this thesis may be retained after development for the purpose of communicating information about the axon environment to the cell bodies. In adult motor neurons, there is evidence that *c*-jun regulates both the survival and axon regeneration capability of these neurons in response to axotomy (Raivich et al., 2004). It is interesting to speculate that adult neurons use the same axon apoptotic signal to activate *c*-jun during development and after axon injury. Using cultured sympathetic neurons as a model, it can be determined if axotomy generates the axon apoptotic signal resulting in *c*-jun activation. The observation that several molecules linked to axon apoptotic signaling (JNKs, *c*-jun, and GSK3) are implicated in the pathology of neurodegenerative diseases such as Parkinson's (Saporito et al., 2000; Willesen et al., 2002) and Alzheimer's (Hernandez and Avila, 2008; Shoji et al., 2000) creates other interesting opportunities for research . The activation of the axon apoptotic signal could be examined in *in vitro* cell culture models of these diseases, starting with models in which neurons are protected from apoptosis by NGF. One potential model is basal forebrain cholinergic neurons that are susceptible to degeneration in Alzheimer's disease (reviewed by Williams et al., 2006).

5.3 Closing remarks

The question of how neurotrophins such as NGF provided at axon terminals mediates the survival of neurons during development has fascinated researchers for decades. Research has come a long way since the discovery of NGF, progressing into the elucidation of the molecular mechanisms underlying NGF-regulated survival. The work in this thesis demonstrates that NGF mediates multiple retrograde signals within the same neuron to regulate neuron survival. The discovery of the axon apoptotic signal holds special significance, because it is the first NGF-regulated retrograde apoptotic signal to be identified. It is clear that effort must be made to continue to study these retrograde signaling mechanisms, since it will not only contribute to an understanding of the process of neuronal development but also to an understanding of the capabilities of neuron signaling in general. This knowledge may lend valuable insight into how neurons respond under a variety of conditions, including those presented by neurotrauma and neurodegenerative diseases.

CHAPTER 6:

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